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Genetic and molecular characterization of maize starch debranching enzymes

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Genetic and molecular characterization of maize starch debranching enzymes

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For the Major Program

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ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
ADP-Glc	Adenosine diphosphoglucose
AGPase	ADP-glucose pyrophosphorylase
bp	Base pair
C	Carbon
DAP	Days after pollination
DBE	Debranching enzyme
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DP	Degree of polymerization
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FACE	Fluorophore associated capillary electrophoresis
Fru-6-P	Fructose-6-phosphate
FBPase	Fructose-1,6-bisphosphatase
Fru	Fructose
FW	Fresh weight
Glc-1-P	Glucose-1-phosphate
Glc-6-P	Glucose-6-phosphate
GBSS	Granule bound starch synthase
Glc	Glucose
h	Hours
HPAEC	High performance anion exchange chromatography
kD	Kilodalton
min	Minutes
MOPS	3-(N-morpholino)propanesulphonic acid

MOS	Maltooligosaccharides
nm	Nanometer
nt	Nucleotide
PCR	Polymerase chain reaction
3-PGA	3-phosophoglycerate
PG	Phytoglycogen
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RNA	Ribonucleic acid
SBE	Starch branching enzyme
± SE	± Standard error of the mean
SEM	Scanning electron microscopy
SDS	Sodium dodecyl sulphate
SS	Soluble starch synthase
Suc	Sucrose
Susy	Sucrose synthase
TEM	Transmission electron microscopy
TIR	Terminal inverted repeat
TPT	Triose phosphate transporter
Tris	3-N-tris(hyrdroxymethyl)aminomethane
TUSC	Trait utility system for corn
U	Units of enzyme activity
UDP-Glc	Uridine diphosphoglucose
UGPase	UDP-glucose pyrophosphorylase
UTR	Untranslated region
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight

ABSTRACT

Starch is the primary carbohydrate reserve of plants, a significant source of food in the human diet, and an important industrial raw material. The arrangement of linear chains and branch linkages within amylopectin is responsible for the highly conserved architecture of the starch granule. This structure allows for efficient packaging of glucose and results in the unique functional properties of starch. It is known that starch synthases and branching enzymes catalyze $\alpha(1\rightarrow4)$ chain elongation and introduction of $\alpha(1\rightarrow6)$ branch linkages, respectively, but studies in several species suggests that starch debranching enzymes (DBE) are also involved in the process of starch synthesis. The overall goal of this research is to determine the role played in maize starch metabolism by each individual DBE. DBEs are broadly classified as either isoamylase- or pullulanase-type based upon their substrate specificity and sequence similarity to defined bacterial proteins. To determine the function(s) of the isoamylase-type DBE, an allelic series of mutations in the gene *Su1* was characterized. The molecular defects of the *su1-* alleles were associated with accumulation of phytoglycogen at the expense of granular starch. The phenotypic severity was also correlated to an alteration in the fine structure of the residual amylopectin. Thus, the SU1 isoamylase-type DBE functions directly in starch synthesis by removing branches from an amylopectin precursor. cDNAs coding for two other isoamylase-type DBE genes, *ZmIso2* and *ZmIso3*, were cloned and characterized with respect to mRNA transcript accumulation. This analysis indicated metabolic specialization for some DBE isoforms either during starch catabolism or anabolism. To investigate the function(s) of the pullulanase-type DBE, reverse genetics was used to identify a transposable element insertion in the maize *Zpu1* gene.

Characterization of this mutation demonstrated that pullulanase-type DBE is important for degradation of both leaf and endosperm starch. ZPU1 also acts during starch synthesis significantly reducing phytoglycogen accumulation in some genetic backgrounds. This suggests that the biosynthetic function of ZPU1 partially overlaps with the biosynthetic function of SU1. Together, these data indicate that isoamylase- and pullulanase-type DBEs are each involved in aspects of both starch synthesis and degradation.

CHAPTER 1. GENERAL INTRODUCTION

Starch is the major carbohydrate storage form in higher plants. It serves a central role in providing energy in the absence of photosynthesis. Starch metabolism in maize, Arabidopsis, and other plants involves nearly thirty enzyme isoforms. Each of these proteins must be regulated for the appropriate timing of starch catabolism and anabolism, synthesizing starch when energy is abundant and degrading starch when energy is needed. Furthermore, proper control of enzyme expression and activity is required for the synthesis of starch with the molecular structure necessary for granule formation. The complexity of this system is likely explained by its fundamental importance in the evolutionary success of plants, for it can be argued that the ability to store large amounts of carbohydrate in insoluble granules provides a distinct advantage over the reduced amount of carbohydrate that can be efficiently stored in soluble glycogen.

The overall goal of this research is to determine the specific function(s) played in starch metabolism by one class of enzymes, starch debranching enzymes (DBEs). A broad body of genetic and biochemical evidence suggests are requirement for these proteins both in starch catabolism and anabolism. A role in starch degradation is clearly anticipated because of the hydrolytic activity of these enzymes. However, plants may have also exploited the hydrolytic activity of DBEs to provide an enzymatic function necessary to facilitate crystallization of glucose polymer into insoluble granules. This review will summarize the substantive aspects of starch metabolism by examining the function of the enzymes involved. Currently available evidence for the proposed dual function of DBEs in starch synthesis and degradation will also be discussed.

Literature Review

Starch Accumulation

Starch accumulates in most plant tissues at some stage of their development. In higher plants, starch is synthesized in plastids in the form of dense, semi-crystalline, water-insoluble granules. These structures vary in size from 1-100 microns, depending on the species and tissue type (Sivak and Preiss, 1998). Starch is usually classified into two types, transient and storage starch, based upon the length of time it is stored. Storage starch serves the long-term needs of the plant and accumulates in reproductive tissues, such as the cereal endosperm or pea embryo, and perenniating organs like potato tubers. In these instances, starch is kept as a reserve by the plant until germination or sprouting and the carbohydrate is used as an energy source prior to attaining photoautotropy. Transient starch accumulates in leaf chloroplasts during the light portion of the diurnal cycle from photosynthetically assimilated carbon. In the subsequent dark period, starch is degraded and converted to sucrose to maintain the supply of carbon to the non-photosynthetic parts of the plant. Starch granules are also synthesized in other plant tissues. In roots and shoots, sedimentation of amyloplasts, the specialized starch-containing plastids, is believed to be the mechanism by which the plant senses gravity, causing a signal transduction cascade leading to the gravitropic response (Chen et al., 1999). The timing of starch mobilization has also been demonstrated to be important for floral transition in Arabidopsis (Eimert et al., 1995; Corbesier et al., 1998). These examples summarily demonstrate that starch is required for the overall development and fitness of the plant.

Commercial Uses of Starch

Globally, the most important sources of starch for human use are the storage organs of maize, potato, rice, and wheat. The commercially useful properties of raw starch isolated from these sources, for example granule size and digestibility, vary considerably (Slattery et al., 2000). Many industrial processes use starch after the granule structure has been partially or completely destroyed by heating in water, i.e. gelatinization. The temperature of gelatinization varies depending on the source of the starch, and is therefore an important consideration in production on an industrial scale. Gelatinized starches from different plant sources form gels or pastes, which vary in viscosity (Ellis et al., 1998). The wide variation in properties of raw starches means that certain starches are preferred for particular end-uses. The useful properties of starch can be further enhanced or altered through physical or chemical modification, producing starches with even more specific properties for use in a range of applications (Ellis et al., 1998).

Starch constitutes approximately 30% (w/w) of the human diet, mainly as a component of foods such as pasta and bread made from food crops such as cereals and tubers (Wang et al., 1998). Isolated starch is also an important ingredient in a range of processed foods where it is used as a thickener, texturizer, stabilizer, and glazing agent. The products of hydrolyzed starch are used in the soft drink industries in the production of sweeteners and syrups (Sivak and Preiss, 1998). Starch is also used extensively in non-food applications. Extracted native and modified starches are used in the production of a wide range of materials such as cosmetics and adhesives. Other non-food applications include use as a sizing, binding and coating agent in the paper and textile industries and in the production of biodegradable plastics and packing materials (Ellis et al., 1998).

It is not completely understood how the molecular structure of the starch granule relates to the properties of different starches during processing. The functional properties of starch depend greatly upon starch structure and composition, which are themselves dependent on the activities of the starch biosynthetic enzymes. The generation of "custom" starches for specific commercial applications could potentially be achieved by manipulation of the expression of the genes that encode the enzymes in the starch synthesis pathway. This could enable the production of starches with increased stability and containing new functional groups, consequently reducing the need for post-harvest modification (Kossmann and Lloyd, 2000). The production of these "designer" starches requires continued research into how starch structure relates to biosynthesis, the regulation of the starch biosynthetic pathway, and the genes that encode the starch synthesizing enzymes.

Starch Composition and Molecular Structure

Starch is composed of two polymers of α-D-glucose (Glc), amylose and amylopectin. Amylopectin is the major polymer, comprising 75% of the relative weight percentage in the mature starch granule. Accordingly, amylose represents the minor polymer comprising 20-30%, depending on the species and tissue type (Buleon et al., 1998; Denyer et al., 2001). Small amounts of phosphate, lipid, and protein are also associated with the starch granule (Kossmann and Lloyd, 2000). Even though it possesses only one kind of monomer unit and two linkage types, the starch granule is organized in hierarchical levels of structure, which contributes to its unique functional properties.

Amylose Component of Starch

Amylose is the minor component of starch and is an essentially linear molecule consisting mainly of long chains of glucose residues (1,000-10,000 residues) linked by α(1→4) glycoside bonds. Amylose from most plant species is essentially unbranched, but may contain a low level (0.2-0.6%) of α(1→6) glucosyl linkages (Hizukuri et al., 1989). The amylose content of transitory starch is usually lower than that of storage starch. For example, starch from Arabidopsis leaves contains 6% amylose when operating in a diurnal cycle of 12 h light. (Zeeman et al., 2002b). Amylose content of storage starch varies depending on the source. Examining the carbohydrate composition from the storage organs of potato, maize and wheat has shown that the amylose content of starch varies between 18 and 36% (Shannon and Garwood, 1984). Amylose content has also been shown to be dependent to some extent on the growing conditions of the plant (Shannon and Garwood, 1984) and on the overall rate of starch synthesis (Clarke et al., 1999; Denyer et al., 2001).

Amylopectin Component of Starch

Amylopectin is the major constituent of starch comprising approximately 75% (w/w) of mature storage starches. Amylopectin molecules are branched and very large. A typical molecule contains between 10,000 and 100,000 glucose units and is approximately 200 to 400 nm long and 10 to 15 nm wide (French, 1984; Shannon and Garwood, 1984). Amylopectin consists primarily of short chains of α(1→4)-linked glucose units joined together with α(1→6) glycosidic linkages (Figure 1.1A).

Within the granule, amylose and amylopectin are organized to form a highly ordered and complex semi-crystalline structure. The degree of crystallinity of the granule varies

depending on the botanical source of the starch. In all cases however, the crystalline nature of the granule can be exclusively attributed to amylopectin, since amylose-free granules of *waxy* mutants of several plant species retain their crystallinity (Smith et al., 1997). The crystalline nature of starch allows for packing of large amounts of Glc in a relatively small space without a requirement for water to be ordered around the individual chains.

The Molecular Structure of the Starch Granule

The currently accepted model for the arrangement of amylopectin within the granule is the cluster model (Figure 1.1B). This model proposes that the α(1→6) linkages are not evenly distributed along the length of the amylopectin molecule but are clustered together at regular 9 nm intervals, separated by regions containing few, if any, branches. The basis for this model arises from the fact that the chain length distribution of amylopectin is polymodal. Amylopectin chains can be divided into three classes according to their length, termed A, B, and C (Buleon et al., 1998). "A chains" are the shortest, containing only 9-16 glucose residues and are not branched. Thus, these chains form the outer branches of a cluster. "B chains" are branched at a C6 position by A chains or other B chains. B chains can be divided into subclasses depending upon the length of the given chain. B1 chains typically contain between 20 and 24 residues. Longer B chains termed B2, B3, and B4 have mean lengths of 41-45, 62-75 and 101-119 glucose residues, respectively. The distribution of the B group chain lengths indicates that these longer chains span more than one cluster and join together successive clusters, i.e. B2 chains span two clusters, B3 chains three clusters and so on (Hizukuri et al., 1989). "C chains" are the only chains in amylopectin with a free reducing end, and thus, only one of these chains exists per molecule. The lengths

of individual glucan chains and the placement of branches determine the primary structure of the starch granule, which leads to further levels of hierarchical organization.

The distribution of chain lengths and the arrangement of amylopectin chains into discrete clusters play a critical role in the overall organization of the starch granule. The

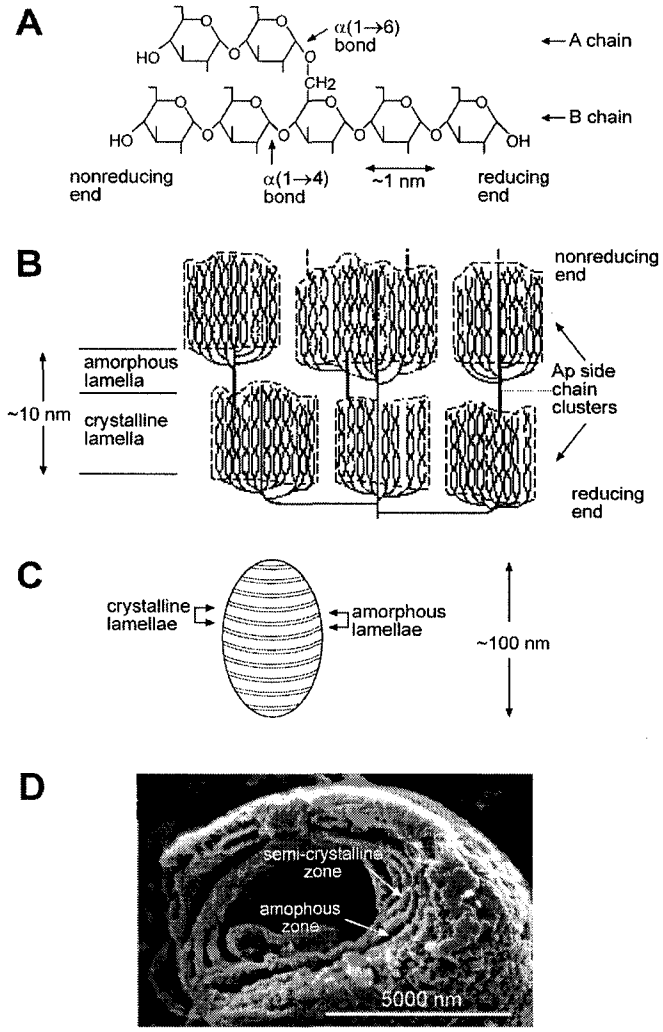

Figure 1.1. Hierarchical levels of starch structure. **A.** Glucose units are joined by α(1→4) and α(1→6) linkages. **B.** Cluster model of amylopectin structure. The boundaries of individual clusters are indicated by the dotted lines. **C.** Diagrammatic representation of a blocklet. **D.** Electron micrograph of a starch granule after partial α-amylolysis showing growth rings. Modified from Myers et al. (2000) and Zeeman et al. (2002b).

amylopectin molecules are arranged radially with their non-reducing ends pointing out towards the surface of the granule (French, 1984). Adjacent A-type chains within the clusters form double helices that pack together in ordered arrays to form the crystalline lamellae of the granule, as illustrated in Figure 1.1B (Gidley and Bulpin, 1987).

Within the granule, the crystalline lamellae containing the double helices of the amylopectin chains alternate with amorphous lamellae in which the amylopectin branch points, i.e. $\alpha(1\rightarrow6)$ linkages, are contained (Jenkins et al., 1993). Observation of granules from a range of species by electron and atomic force microscopy suggests a further level of organization. The crystalline and amorphous lamellae appear to be arranged within a small, stacked, spherical "blocklet" (Figure 1.1C). The diameter of these blocklets ranges from 20 to 500 nm depending on the botanical source of the starch and the location of the blocklet within the granule (Gallant et al., 1997).

Blocklets that differ in size and/or orientation contribute to distinct areas within the granule, termed amorphous and semi-crystalline zones or shells (Figure 1.1D). One amorphous and one semi-crystalline zone constitute what is known as a growth ring. Growth rings are the largest-scale level of organization within the granule. An individual ring is on the order of several hundred nanometers making it possible to observe growth rings by electron microscopy after mild treatment of the granule with starch degrading enzymes (Buleon et al., 1998).

Amylose molecules exist in the granule in the form of single helices (Gidley and Bociek, 1988). These helices are thought to be interspersed with amylopectin and primarily located in the amorphous lamellae of the amylopectin matrix (Buleon et al., 1998; Kossmann and Lloyd, 2000). Jenkins and Donald (1995) used X-ray scattering to investigate the effect

of varying amylose content on the structure of starch granules from maize, potato, barley and pea. The results from this study suggest that amylose slightly alters the arrangement of amylopectin clusters, indicating that some amylose is also present within the crystalline lamellae.

The Pathway of Starch Synthesis

To attain the structure of the granule just described, the enzymes involved in starch synthesis must be properly regulated. Starch synthesis occurs in nearly every plant tissue at some stage of development, but starch is most abundant in leaves and storage organs, such as endosperm and tuber. In leaves, starch is synthesized during the day, when energy is abundant for the fixing of carbon, and degraded at night when energy is required. In storage organs, starch is synthesized continuously from carbon imported from photosynthesizing tissues. Irregardless of the tissue, the committed step of the starch biosynthetic pathway is the production of the Glc donor compound, ADP-Glc. This section will discuss the generation of ADP-Glc and describe the various enzymes of amylose and amylopectin synthesis.

ADP-Glucose Synthesis in the Chloroplast

ADP-Glc in the chloroplast is synthesized directly from the intermediates of the photosynthetic carbon reduction cycle (Calvin cycle). One of these intermediates, Fru-6-P, can be readily converted to Glc-1-P by chloroplastic phosphoglucomutase and isomerase (Trethewey and Smith, 2000). Glc-1-P and ATP then serve as the substrates for ADP-Glc Pyrophosphorylase (AGPase) to synthesize ADP-Glc. In the chloroplast, this enzyme is under tight allosteric control (discussed in a following section), only synthesizing starch

when photosynthetic carbon reduction cycle intermediates and energy are abundant. AGPase in heterotropic tissues is also under allosteric control, but may not be as sensitive to effectors (Hannah et al., 1995).

Starch and sucrose metabolism is made more complex in maize and other C4 plants because mesophyll and bundle sheath cells are each specialized for photosynthesis and carbon fixation. CO_2 is taken up by the mesophyll cells and converted to by malate, which is exported to the bundle sheath cells. In the bundle sheath, NAD-malic enzyme releases CO_2 from malate, generating pyruvate. The lignification of the bundle sheath cell wall concentrates the CO_2 in these cells (Lunn and Furbank, 1997). Thus, the carboxylation reaction of Rubisco is preferred over the oxygenation reaction, which results in an increased photosynthetic efficiency. Starch and Suc metabolism is altered in C4 plants because of the specialized anatomy. 3-PGA is exported from the bundle sheath cells to the mesophyll where sucrose synthesis occurs. Starch synthesis occurs solely in the bundle sheath chloroplast from Calvin cycle intermediates (Lunn and Furbank, 1997).

Sucrose Metabolism in the Cytosol of Storage Organs

For starch synthesis in storage organs, the source of carbon is Suc, which is exported from photosynthesizing tissue. Within heterotrophic tissues, metabolic plasticity allows for multiple routes of flux from Suc to ADP-Glc (Figure 1.2). Genetic studies have shown that the initial metabolism of Suc in the cereal endosperm is dependent on invertase and sucrose synthase (Susy). The importance of these steps in the biochemical pathway has been demonstrated by study of maize *sh1* and *mn1* mutants, (Cheng et al., 1996; Chourey et al., 1998). Loss of Susy in *sh1* kernels causes a 22% reduction in the amount of total starch,

and mutation of cell wall invertase in *mn1* causes a 70% reduction in seed weight. Invertase (β-D-fructofuranosidase) cleaves Suc to Glc and Fru, whereas Susy generates UDP-Glc and Fru, thereby conserving one high-energy bond (Figure 1.2). Ultimately, the hexose phosphate Glc-1-P is used as a substrate by AGPase to generate ADP-Glc, requiring one ATP. Isolated plastids have been shown to uptake Glc-P or ADP-Glc directly.

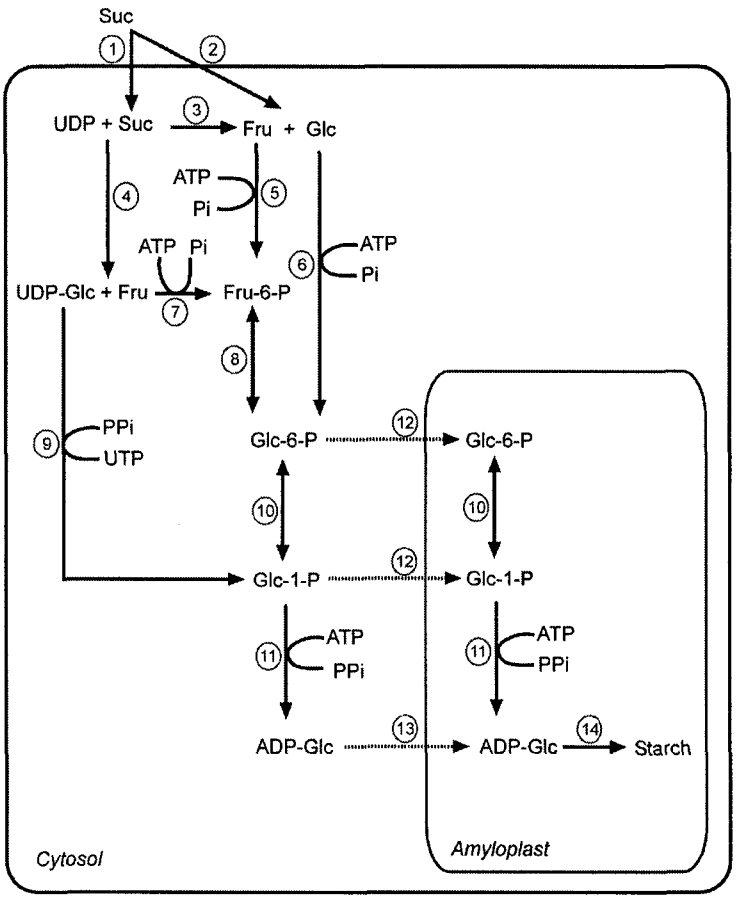

Figure 1.2. Sucrose metabolism in endosperm tissue. The major metabolites and enzymes involved in the conversion of sucrose to starch in cereal endopserm. (1) Sucrose transporter. (2) Cell wall invertase. (3) Soluble invertase. (4) Sucrose synthase. (5, 7) Fructokinase. (6) Glucokinase. (8) Isomerase. (9) UDP-Glc pPyrophosphorlyase. (10) Phophoglucomutase. (11) ADP-Glc pyrophosphorylase. (12) Hexose phosphate transporter. (13) Adenylate transporter. (14) SS, SBE, DBE.

Recent studies have shown that there is significant reprocessing of Glc prior to its incorporation in ADP-Glc, through glycolysis, gluconeogenesis, and the pentose phosphate pathways (Glawishnig et al., 2002). Thus, the flux of carbon through the pathway illustrated in Figure 1.2 is likely not direct.

Transport of Carbon into the Amyloplast

The major form in which carbon enters the plastid from the cytosol is hexose phosphate, although the nature of this compound may vary. The form of hexose phosphate that is transported into the plastid and used for starch synthesis has been determined in a number of species from measurements conducted on isolated, intact, and metabolically active amyloplasts. Glc-6-P was shown to be the compound imported for starch synthesis in amyloplasts from pea embryo (Hill and Smith, 1991). Glc-1-P as opposed to Glc-6-P is used for starch synthesis in amyloplasts from wheat endosperm (Tyson and ap Rees, 1988; Tetlow et al., 1994). In amyloplasts from maize endosperm, both Glc-6-P and Glc-1-P are imported and can serve as precursors for starch synthesis (Neuhaus et al., 1993).

The transporter(s) that mediate the import of hexose phosphates across the inner amyloplast membrane is generally predicted to be a hexose phosphate exchanger, analogous to the triose phosphate exchanger in chloroplasts. The removal of Pi from the plastid is of critical importance to the regulation of starch synthesis, as Pi is generally observed to be a potent inhibitor of AGPase (Ghosh and Preiss, 1966). Mohlmann et al. (1997) demonstrated that maize amyloplasts are able to transport Glc-6-P but not Glc-1-P in counter exchange for Pi. Molecular evidence for the presence of a Glc-6-P/Pi translocator in maize amyloplasts has been obtained by Kammerer et al. (1998). The authors reported that a 31 kDa protein

purified from maize endosperm mediates the import of Glc-6-P into the amyloplast and the export of Pi in a ratio of 1:1. The sequence of this peptide was then used to isolate corresponding cDNA clones from pea roots, potato tubers and cauliflower buds suggesting that presence of a Glc-phosphate transporter is widespread in heterotrophic tissues (Kammerer et al., 1998). However, Tetlow et al. (1996) have shown that proteins from wheat amyloplasts counter-exchange Pi and G-1-P/G-6-P with equal efficiency, suggesting the hexose phosphate/Pi exchanger in wheat does not share the substrate specificity with the transporter identified in maize.

Evidence also exists from studies in several plant species that ADP-Glc synthesized in the cytosol is imported directly into the plastid via an adenylate transporter (Pozueta-Romero et al., 1991a; Pozueta-Romero et al., 1991b). Mohlmann et al. (1997) demonstrated that maize endosperm amyloplasts are capable of importing ADP-Glc at greater rates than Glc-6-P. Uptake of ADP-Glc was shown to be in counter-exchange for ADP and AMP. A transporter capable of mediating the import of ADP-Glc into the amyloplast has been identified in developing maize endosperm. It has been suggested that this protein is encoded by the *Brittle1* (*Bt1*) gene, which shows sequence similarity to known adenylate transporters (Cao et al., 1995). Evidence that the BT1 protein is involved in the import of ADP-Glc into the amyloplast for starch synthesis comes from studies of *bt1* mutants. Shannon et al. (1996) have shown that the maize *bt1* mutants display a large reduction in ADP-Glc transport across the amyloplast membrane and a five-fold decrease in the rate of starch synthesis. These also accumulate high levels of ADP-Glc (13-17 fold higher than wild-type), consistent with the idea that the sugar nucleotide is synthesized in the cytosol but cannot be transported into the amyloplast (Shannon et al., 1996).

Localization of ADP-Glucose Pyrophosphorlyase

For many years, it was assumed that the activity of AGPase was confined entirely to the plastid, and thus all ADP-Glc was synthesized in this compartment. There is now good evidence that in the endosperm of the cereals barley (Thorbjornsen et al., 1996; Johnson et al., 2003), maize (Hannah et al., 2001), and wheat (Beckles et al., 2001; Burton et al., 2002a) in particular, there are two forms of AGPase, one cytosolic and one plastidial. Furthermore, the authors showed that in these tissues the cytosolic form of the enzyme accounts for most of the AGPase activity. In all vegetative tissues and in the heterotrophic tissues of most other species, all of the AGPase activity is confined to the plastid (Beckles et al., 2001).

AGPase is a heterotetrameric enzyme, consisting of two large and two small subunits. In maize endosperm, the cytosolic forms of these polypeptides are coded for by the *Shrunken2* (*Sh2*) and *Brittle2* (*Bt2*) genes, respectively (Bae et al., 1990; Bhave et al., 1990). The two subunits have been separately conserved throughout the evolution of plants, and both are required for ADP-Glc production. However, the sequences of the small subunit genes possess less heterogeneity than those of the large subunit genes (Hannah et al., 2001; Johnson et al., 2003). When in complex, the large subunit is speculated to be regulatory in nature, whereas the small subunit is primarily involved in catalysis. However, under certain conditions the large subunit may show catalytic activity (Smith et al., 1997), so it is likely that both subunits are involved in dual aspects of catalysis and regulation.

Control of ADP-Glucose Pyrophosphorylase

Overwhelming evidence suggests that AGPase provides the only route of ADP-Glc synthesis in both photosynthetic and non-photosynthetic tissues. In mutant and transgenic plants where the activity of AGPase is reduced, a large decrease in starch accumulation is also observed. In the endosperms of the *shrunken2* (*sh2*-) and *brittle2* (*bt2*-) mutants of maize, the AGPase activity is reduced by 90 to 95% and the starch content is reduced by 70 to 75% compared to wild-type endosperms (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). Similarly, Arabidopsis leaves with low or no AGPase activity accumulate a reduced amount of starch (Lin et al., 1988a, b) and transgenic potato tubers expressing only low levels of AGPase (2-17% of wild type activity) display a decrease in starch content to 65-95% of wild-type levels (Muller-Rober et al., 1992). Conversely, a maize *sh2* mutant proposed to cause increased AGPase activity results in enhanced starch synthesis as evidenced by added seed weight (Giroux et al., 1996).

AGPase purified from a wide range of species is allosterically regulated. It is activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi) (Sivak and Preiss, 1998). AGPase from both photosynthetic and many heterotrophic tissues is strongly regulated by these factors. However, studies in barley and maize show that the major cytosolic forms of AGPase in the endosperm are not regulated significantly by the ratio of 3-PGA: Pi (Kleczkowski et al., 1993; Hannah et al., 1995). These results suggest that the mechanisms of regulation of starch synthesis in tissues where ADP-Glc is predominantly synthesized in the cytosol may differ significantly from that described above for other plants where ADP-Glc is synthesized exclusively in the plastid.

These regulators are notable since AGPase exerts a signficant degree of control over the overall pathway of starch synthesis. The control exerted by an enzyme in a pathway may be assessed through the application of metabolic control analysis (Neuhaus and Stitt, 1990; ap Rees and Hill, 1994; Stitt, 1995). Metabolic control analysis uses flux control coefficients to quantify the degree of control an enzyme exerts. These values are obtained by making a small change in the activity of the enzyme (for example through the expression of antisense RNA) and measuring the resultant change in flux through the pathway. Geigenberger et al. (1999) and Sweetlove et al. (1999) have estimated that the flux control coefficient of AGPase on starch accumulation in intact potato plants to be between 0.3 and 0.5. Comparison between this and flux control coefficients of other starch metabolizing enzymes suggest that AGPase may perform the rate-limiting step of starch synthesis.

Soluble Starch Synthase

Starch synthases serve to elongate glucan chains, by adding the glucosyl moiety from the compound ADP-Glc to the growing polyglucan chain (Figure 1.3A). The precise mechanism for the action of these enzymes is unknown. The currently accepted model is that these enzymes work processively by adding single glucose units to the non-reducing end of a pre-formed glucan primer, i.e. the primer-dependent mechanism (Figure 1.3A). Alternatively, a covalent complex may form between the glucose residue and the enzyme. Elongation then proceeds via a two-point insertion mechanism (Mukerjea et al., 2002). Starch synthase activity is present in the soluble phase of the plastid and bound to the granule. The activities primarily detected in the soluble phase are responsible for the synthesis of amylopectin.

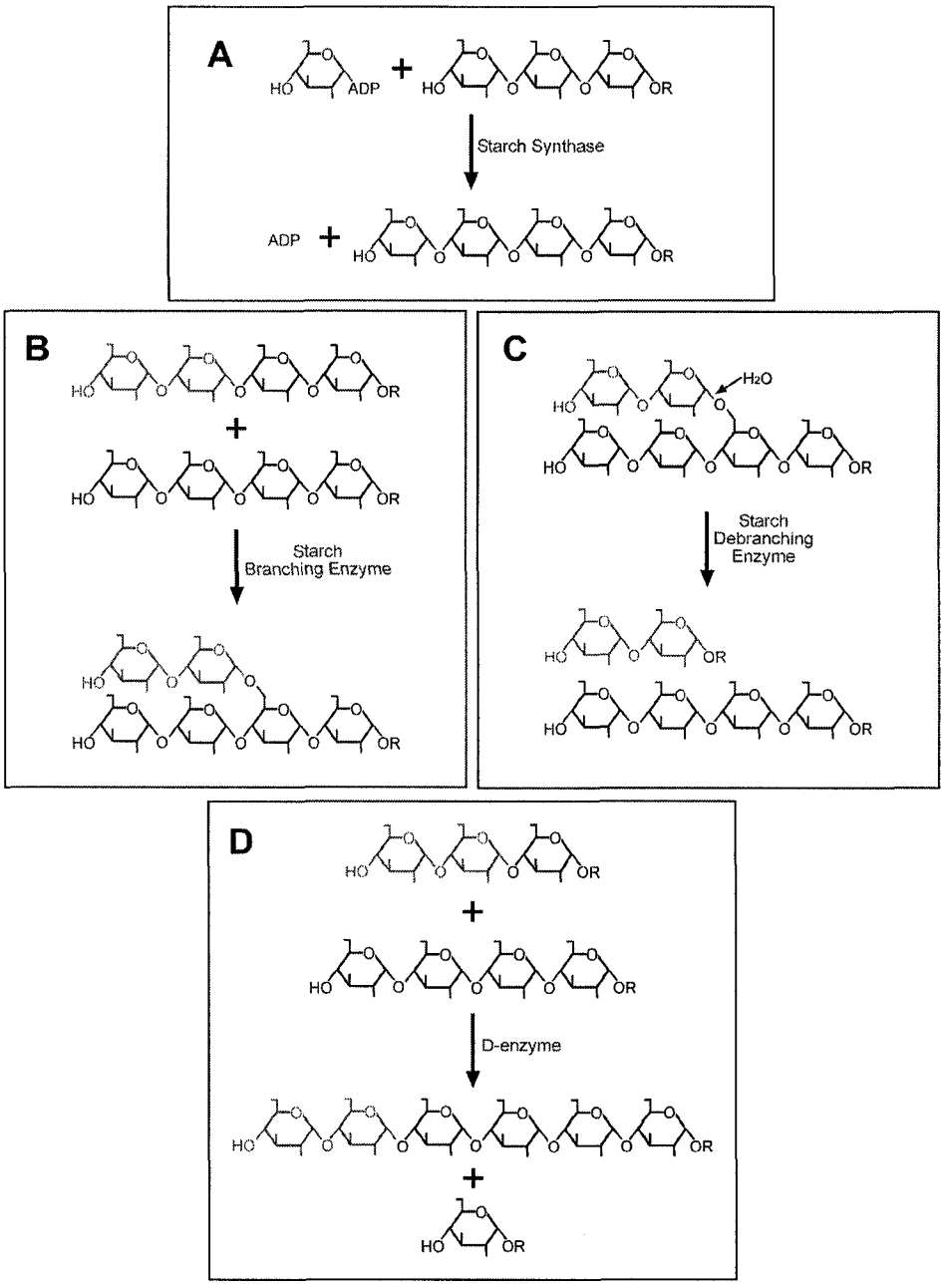

Figure 1.3. Reactions catalyzed by starch metabolizing enzymes. Representative chemical reactions are shown with transferred glucose residues indicated in red. **A.** Starch synthase (SS). **B.** Starch branching enzyme (SBE). **C.** Starch debranching enzyme (DBE). **D.** Disproportionating enzyme (D-enzyme).

Multiple isoforms of soluble starch synthase (SS) have been identified at both the protein and molecular level. From these studies three distinct classes of SS isoforms, termed SSI, SSII, SSIII have been identified from a range of species and grouped the basis of activity peaks following anion exchange chromatography of protein extracts. The first member of the SSI class was identified in rice (Baba et al., 1993). On the basis of similarities in their amino acid sequence, SSI isoforms have subsequently been identified in maize (Knight et al., 1998) and other species. A distinct class of SSII isoforms has been discovered in maize (Imparl-Radosevich et al., 1999), pea (Dry et al., 1992), potato (Edwards et al., 1999a), and wheat (Li et al., 1999). The third class of SS isoforms, SSIII, was identified in potato (Marshall et al., 1996), maize (Gao et al., 1998) and wheat (Li et al., 2000). The Arabidopsis genome sequence reveals that this plant has at least five genes encoding SS, including genes coding for proteins very similar to SSI, SSII and SSIII. It therefore seems likely that most species possess three to five distinct isoforms of SS.

In different species and organs, the isoforms of SS contribute to the total SS activity to different extents. For example, in pea embryo, SSII accounts for 60-70% of the soluble activity (Denyer et al., 1993). However, in potato tuber SSII contributes a maximum of only 15% to the total SS activity (Edwards et al., 1995), and the major isoform is SSIII, which accounts for approximately 80% of the activity in the soluble fraction (Marshall et al., 1996). In maize endosperm SSIII accounts for 20-30% of the soluble activity, and SSI has been shown to be the major SS isoform (Cao et al., 1999).

Some isoforms of soluble SS have been shown to be associated with the granule. The SSII isoform of pea embryo (Denyer et al., 1993), SSII and SSIII isoforms of potato tuber (Marshall et al., 1996), SSI isoform of maize (Mu-Foster et al., 1996), and the SSII

isoform of wheat (Li et al., 1999) have been identified in both the soluble and granule bound fractions. Denyer et al. (1993) proposed that the soluble proteins may become entrapped within the granule as amylopectin chains on the granule surface are elongated and crystallize. Also, Commuri and Keeling (2001) found that maize SSI has a high affinity for chains greater than 20 DP. Even though the enzyme does not appear to elongate such chains, the binding of the protein may explain why it attaches to the granule. The contribution of the granule bound fraction of these isoforms to amylopectin synthesis remains to be discerned.

Studies of several species of plants with altered SS activity suggest that the different isoforms of SS may play distinct roles in the formation of amylopectin. To examine the specific role played in starch biosynthesis by the SSI isoform, Commuri and Keeling (2001) investigated the kinetic properties of the maize enzyme on various substrates including glycogen, amylose, and amylopectin. They have suggested that SSI is responsible for elongation of short A-type and B1 chains to a critical length, where catalysis is no longer suitable. Branching would then occur and elongation would continue or crystallization would fix that particular chain in position within the granule. The authors suggest that SSI plays a critical role in organizing each cluster. Thus, loss of SSI would profoundly alter amylopectin structure, and they offer that such a mutant would be starchless, explaining why there has been no mutant of SSI identified in classical genetic screens.

Developing pea embryos carrying a mutation at the *rug5* locus, which has been shown to encode the SSII isoform, contain little or no SSII protein and amylopectin with an altered structure. The amylopectin contains an increased number of long (B4) chains and short (A and B1) chains and a decreased number of intermediate length (B2, B3) chains, suggesting that in pea SSII is responsible for the production of intermediate length chains

(Craig et al., 1998). Similarly in *Chlamydomonas*, a mutation at the *st-3* locus, which causes in a decrease in the activity of one of the isoforms of starch synthase, results in the synthesis of amylopectin enriched in very short chains and depleted in intermediate length chains, suggesting that the ST-3 protein is responsible for the production of the latter (Fontaine et al., 1993). Finally, studies of mutants in the maize *sugary2* (*su2*) gene, which codes for the SSIIa isoform (Zhang et al., manuscript in preparation), have indicated a relative decrease in short chain lengths and an increase in longer chains.

Evidence for the function of the SSIII isoform was revealed by studies of the maize *Dull1* (*Du1*) gene. The *du1* mutants were shown to be deficient in SSIII activity (Gao et al., 1998; Cao et al., 1999). Endosperm starches from *du1* plants was shown to more highly branched and possess more A- and short B-type chains than wild-type, suggesting that this enzyme isoform is responsible for the synthesis of longer B chain material (Wang et al., 1993).

However, it is an oversimplification to assign the synthesis of a particular chain length to a specific SS isoform. Studies in potato have shown that the precise role played by a particular isoform of starch synthase in the synthesis of amylopectin is dependent on the other isoforms present. Transgenic potatoes expressing antisense RNA for either SSII and/or SSIII were used to investigate the interactions of isoforms of SS. Edwards et al. (1999a) showed that SSII, SSIII and SSII/SSIII antisense tubers all produce amylopectin enriched in shorter chains (6-12 glucose units) and depleted in intermediate length chains (15-35 glucose units) relative to the wild-type. However, the precise pattern of enrichment and depletion differed significantly between the three sets of plants. For example, amylopectin from SSII antisense tubers contained a higher frequency of chains of DP 8-12 and a reduced amount of

chains of DP 15-25. SSIII antisense tubers produced amylopectin considerably enriched in chains of DP 6 but changes to the intermediate length chains were less pronounced. Amylopectin from SSII/SSIII antisense tubers contained a higher frequency of chains of DP 7 and 13 and a strong depletion in chains greater than DP 15. Lloyd et al. (1999) have conducted a similar study and again reported a general increase the frequency of chains of DP 6 to15 in starch from all three sets of plants and a decrease in the frequency of chains DP between 20 and 40. In both studies the shift in production of long chains to short chains was greater in amylopectin from plants with reduced SSIII activity compared to those with reduced SSII, consistent with SSIII being the major isoform of soluble SS in potato.

The difference in chain length distribution between amylopectin from SSII and SSIII antisense tubers agree with the earlier studies conducted on mutants of pea, maize, and *Chlamydomonas* in that they suggest that the individual isoforms may play different roles in the synthesis of amylopectin. However, the changes in chain length distribution observed in amylopectin from the tubers of plants in which the activity of both SSII and SSIII were reduced simultaneously were not simply the sum of the changes observed in amylopectin from plants where SSII and SSIII were reduced individually. These results suggest that the SSII and SSIII isoforms of SS do not act independently during the synthesis of amylopectin but act in a synergistic manner. This synergy is most likely to arise because the product of one isoform provides a substrate for the other isoforms. The changes in the relative activities of one or more isoforms in the transgenic tubers would cause changes in the substrate and therefore the product of the other isoforms (Edwards et al., 1999a; Smith, 1999).

Granule-Bound Starch Synthase

The synthesis of amylose is the specific function of an exclusively granule-bound isoform of starch synthase (granule-bound starch synthase I; GBSSI). Starch granules from mutants deficient in this isoform contain low or undetectable quantities of amylose. This phenotype has been observed in GBSSI mutants from a wide range of species such as the *waxy* (*wx*) mutants of maize (Tsai, 1974), rice (Murata et al., 1965), and wheat (Nakamura et al., 1995).

In all these cases where the activity of GBSSI is reduced or eliminated, the soluble isoforms of SS appear to be unable to contribute to the synthesis of amylose. It has been suggested that either the location of GBSSI, buried within the granule, and/or specific kinetic properties of the enzyme could be responsible for the unique ability of GBSSI to synthesize amylose. Evidence to support these hypotheses and potential mechanisms for amylose synthesis are described below.

The manner in which GBSSI elongates small, soluble MOS that diffuse into the granule from the stroma may be responsible for the unique ability of the enzyme to synthesize amylose (Denyer et al., 1999; Edwards et al., 1999b; Edwards et al., 2002). It was suggested that the processive action of GBSSI leads to the production of long linear glucan chains from ADP-Glc and small, soluble MOS. These long chains are too large to diffuse readily out of the granule matrix. Thus, they remain trapped within the granule, and comprise the amylose portion of starch. The soluble isoforms of SS are unable to produce chains of sufficient length to prevent them from diffusing out of the granule matrix into the stroma. Commuri and Keeling (2001) have shown that the SSI enzyme of maize is unable to efficiently catalyze elongation beyond DP ~20 *in vitro*, and could not create the long, linear

chains of amylose. The MOS-priming model is supported by *in vivo* studies of amylose synthesis. Zeeman et al. (2002a) noted that the Arabidopsis *dpe1* mutant, which accumulates significant levels of MOS, displays a higher percentage of amylose in the leaf. Furthermore, these authors used pulse-chase labelling experiments to show that there was no transfer of ^{14}C-Glc from the amylopectin fraction to the amylose fraction in Arabidopsis plants.

This data contradicts an alternative mechanism for the synthesis of amylose that has been proposed by van de Wal et al. (1998). The authors suggest that amylose is synthesized by the extension and cleavage of amylopectin rather than from the elongation of small, soluble MOS. The evidence to support this hypothesis comes from ^{14}C incorporation experiments conducted on isolated *Chlamydomonas* starch granules. Pulse-chase experiments revealed that the ^{14}C incorporated from ADP-^{14}C-Glc moved progressively from the amylopectin component to the amylose component of the starch. These results led van de Wal et al. (1998) to suggest that GBSSI continuously uses amylopectin as a primer, elongating long outer chains until a sufficient length is reached and a cleavage event occurs. The mechanism of cleavage is unknown, but could be performed by a starch hydrolase located within the granule.

This mechanism is supported to some extent by various studies which demonstrate that GBSSI incorporates glucose into amylopectin as well as amylose. First, studies conducted on isolated granules from potato tuber (Denyer et al., 1996b) showed that GBSSI incorporates ^{14}C from ADP-^{14}C-Glc into long chain amylopectin in the absence of MOS. Secondly, the *sta2* mutants of *Chlamydomonas*, which display reduced activity of GBSSI, not only produce starch with decreased levels of amylose but also synthesize modified amylopectin, which lacks long chains (Delrue et al., 1992). These observations were

supported by the genetic studies of Maddelein et al. (1994), which showed that in specific mutant backgrounds GBSSI controlled the amount and structure of the long amylopectin chains. This proposed mechanism of amylose synthesis is in contrast to that proposed by Denyer et al. (1999), described earlier in this section, which suggests that amylose synthesis proceeds via the processive elongation of MOS, promoted by the interaction of GBSSI with the amylopectin matrix. Thus, evidence to conclusively establish a general mechanism for amylose synthesis during granule formation is lacking.

Branching Enzymes

The $\alpha(1\rightarrow6)$ branch points of amylopectin are introduced by starch branching enzyme (SBE). SBE catalyzes the breakage of $\alpha(1\rightarrow4)$ bonds and the transfer of the oligosaccharide to a C6 position on the original or an adjacent glucan chain (Figure 1.3B) (Borovsky et al., 1979a; Borovsky et al., 1979b). As in the case of the SSs, multiple isoforms of SBE have been identified in a variety of species. SBE isoforms can be divided into two broad classes, termed A and B on the basis of their antigenic properties and amino acid sequence (Burton et al., 1995). Members of the A-class of SBE contain an N-terminal sequence that is absent from the B-class isoforms (Burton et al., 1995; Gao et al., 1997). In maize and rice for example, three isoforms of SBE have been identified; two class A isoforms SBEIIa and SBEIIb, and one class B isoform, SBEI (Gao et al., 1996, 1997). The genes for the two A isoforms are differentially expressed; IIa is expressed in the vegetative tissues and endosperm, whereas IIb is expressed exclusively in endosperm (Gao et al., 1996, 1997). SBE of both classes are found in both the soluble and granule bound fractions in a variety of species. For example, in pea SBEI and SBEII have been found in both the soluble

and granule bound fractions (Denyer et al., 1993). Granule association of SBE has also been reported in maize (Mu-Foster et al., 1996) and wheat (Rahman et al., 1995).

Biochemical studies have suggested that the SBE II (A-class) and SBE I (B-class) isoforms in maize have different substrate specificities. The kinetic properties of the maize isoforms have been investigated *in vitro* using both purified and recombinant proteins (Guan, 1993; Takeda et al., 1993). Both studies concluded that the A-class isoforms displayed a lower affinity for amylose and a higher affinity for amylopectin than the B-class isoform. Takeda et al. (1993) also demonstrated that the B-class isoform preferentially transferred longer chains than the A-class isoform. These observations lead the authors to suggest that the A- and B-class isoforms play distinct roles in the synthesis of amylopectin. The A-class isoforms were proposed to be responsible for generating the short A- and B1-chains that lie within one cluster, and the B-class is isoform responsible for initiating the longer B-chains, which span multiple clusters.

Rice and maize endosperms carrying a mutation at the *amylose extender* (*ae*) locus, in the gene that encodes SBEIIb (A-class; (Stinard et al., 1993; Fisher et al., 1996)), and pea embryos carrying a mutation at the *rugosus* (*r*) locus, in the gene that encodes SBEI (A-class isoform; (Bhattacharyya et al., 1990; Smith, 1988)), contain amylopectin with an increased proportion of long chains, and an increase in the level of apparent amylose. Similarly, leaf starch of the SBEIIa mutant of maize exhibits an increased proportion of long chains compared to the wild-type (Blauth et al., 2001). These observations are consistent with the biochemical characterization of these enzymes.

Genetic evidence suggests that the biochemical data does not completely describe the function of these enzymes. The *ae* and *r* mutations also result in decreased accumulation of

starch (Martin and Smith, 1995). It is possible that the changes observed in amylopectin structure resulted from an overall decrease in SBE activity relative to SS activity and from changes in the rate of starch synthesis. To control for these effects, Tomlinson et al. (1997) looked at starch synthesis in pea leaves. The *r* mutation causes a 10-fold decrease in the activity of the A-class isoform in leaves but unlike in the embryo, the mutation has little or no effect on the rate of starch synthesis. The authors reasoned that the specific effect of the loss of the A-class isoform on the structure of amylopectin could then be defined unambiguously in the leaf. Analysis of the chain length distribution of amylopectin showed that there was an increase in the frequency of longer chains relative to shorter chains from the leaves of the *r* mutant compared to the wild type. Genetic studies have not revealed a function for the B-class SBE isoform. The SBEI mutant of maize has no effect on amylopectin structure, in either leaf or endosperm starch (Blauth et al., 2002).

Evidence of a functional interaction between branching enzymes has been suggested by Seo et al. (2002). In this study, maize SBE isoforms SBEI, SBEIIa, and SBEIIb were expressed in *Saccharomyces cerevisiae* with the endogenous glycogen synthase (GS). The authors found that there was a particular kind of synergism between these enzymes to generate novel glucans. Specifically, SBE was required for optimal stimulation of GS. In turn, GS activity was required for any detectable activity of the SBE. The authors also demonstrated that SBEI activity was dependent on the presence of SBEIIa and SBEIIb, suggesting that the substrate for SBEI is the product of SBEIIa and SBEIIb branching. These data point toward a sequential series of glucan construction beginning with branching by SBEIIa/SBEIIb, followed by chain elongation, and subsequent branching by SBEI (Seo et al., 2002). In summary, the evidence described in this section suggests that the A-class and

B-class isoforms of SBE make different contributions to the synthesis of amylopectin. The results also indicate that like the SS isoforms, the isoforms of SBE may well act in a synergistic manner.

Debranching Enzymes

Starch debranching enzymes, i.e. α(1→6) glucan hydrolases, cleave α(1→6) branch linkages in glucose polymers (Figure 1.3C). The hydrolytic activity of these proteins points toward a *prima facie* role in starch degradation, but genetic and biochemical studies in many plant species implies that these enzymes function in starch biosynthesis as well. The specific function of DBEs during the biosynthesis of amylopectin will be discussed in a following section and is the subject of subsequent chapters of this dissertation.

Disproportionating Enzyme

Disproportionating enzyme (D-enzyme) is an α-glucan transferase, which cleaves an α(1→4) linkage and transfers the reducing end to another glucan chain, creating a new α(1→4) linkage, releasing Glc (Figure 1.3D). Evidence from *Chlamydomonas* suggests that this enzyme may be involved in starch biosynthesis (Colleoni et al., 1999b; Colleoni et al., 1999a). The *Chlamydomonas* D-enzyme mutant *sta11* shows a marked decrease in the amount of total starch compared to wild-type and a ten-fold increase in MOS content, even though no effects on other starch metabolizing enzymes are observed. Furthermore, the residual starch of *sta11* cells displays significant alterations, visible at the level of granule structure and chain length distribution (Colleoni et al., 1999b). The mechanism by which D-enzyme changes the amylopectin structure is not clear. D-enzyme may modify an amylopectin precursor polymer directly, or the abnormal metabolism of MOS may affect

other enzymes which are directly involved in the determination of the amylopectin fine structure. Enzymatic characterization of the *Chlamydomonas* D-enzyme showed that the protein was capable of transferring Glc from donor oligosaccharides to the outer chains of amylopectin (Colleoni et al., 1999a). Thus, the model of D-enzyme function in starch biosynthesis is supported by the genetic and biochemical data, but pleiotropic effects resulting from abnormal MOS metabolism cannot be excluded.

The model suggested by the *Chlamydomonas* study is inconsistent with the proposed function of D-enzyme in Arabidopsis (Critchley et al., 2001). Like the *sta11* mutant, the Arabidopsis D-enzyme mutant *dpe1* is impaired in its ability to synthesize starch during the day in plants operating under normal diurnal conditions (Critchley et al., 2001). However, when the dark period is extended (to allow for the degradation of all the starch and MOS) and the plants are placed back into the light for one cycle, the plants synthesize starch normally. Thus, the authors speculate that the starch biosynthetic defect in Arabidopsis is caused by interference from the high levels of MOS and not due to a direct role for D-enzyme in starch biosynthesis. The *dpe1* mutation does have profound effects on starch degradation (Critchley et al., 2001), and these will be discussed in the next section. Consequently, the role of D-enzyme in the process of starch synthesis cannot be generalized among species. It may be that the requirement for D-enzyme in starch biosynthesis differs between *Chlamydomonas* and higher plants, or the function of D-enzyme in starch synthesis is substituted by another enzyme in Arabidopsis.

The Pathway of Starch Degradation

In the absence of photosynthesis, the majority of a plant cell's energy is supplied by mitochondrial respiration. This process requires an oxidizable substance, such as carbohydrate. Starch, the primary carbohydrate storage form, is degraded when carbon is required by the plant, either during the dark portion of the diurnal cycle of leaves or during germination of seeds. Starch can be degraded by hydrolysis or phosphorlysis, through the cooperative action of several enzymes (Figure 1.4). The ultimate product of the hydrolytic

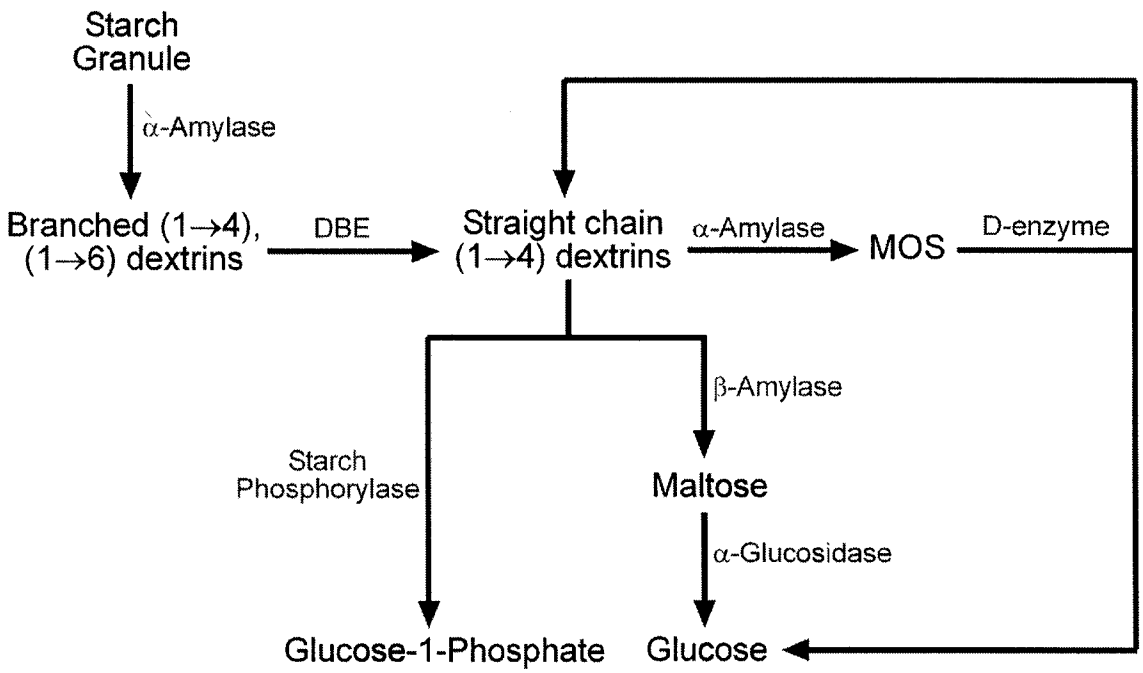

Figure 1.4. Pathway of starch degradation. The principal enzymes and products of starch degradation are shown.

pathway is Glc, whereas the product of phosphorolysis is Glc-1-P. Like the starch biosynthetic enzymes, several isoforms exist for each of the degradative enzymes. For instance, the Arabidopsis genome contains three predicted α-amylases, nine β-amylases, two starch phosphorylases, two D-enzymes, and four debranching enzymes. The specific expression pattern of the genes is not known, nor is it known the precise nature of each gene product's contribution to the overall process of starch degradation. Most studies of this process have involved *in vitro* characterization of individual enzymes, such as substrate specificity and activity. This section will examine the enzymes of starch degradation and their individual and synergistic contribution to this metabolic process.

Control of Starch Degradation

The control of starch degradation varies depending on the tissue type. In the cereal endosperm, expression and activity of starch hydrolytic enzymes are regulated by the hormone giberellic acid (GA). GA is released by the embryo and this compound stimulates the aleurone layer of the endosperm to secrete a wide array of proteins, including amylases as well as proteases, which activate other starch degradative proteins by processing the proenzymes to make the active enzyme (Beck and Ziegler, 1989). In chloroplasts, the timing of starch degradation may be controlled at the transcriptional level (Trethewey and Smith, 2000); other enzymes may be controlled post-translationally, either by allosteric regulation, redox modification, or responses to change in pH. In the case of starch phosphorylase, allosteric regulation is clearly important, since the enzyme is strongly activated by P_i (Stitt and Steup, 1985), which would increase when the overall energy level of the cell is low. Other enzymes such as β-amylase and the DBE pullulanase, may be activated up to two-fold

when reduced by thioredoxin (Cho et al., 1999; Wu et al., 2002; Balmer et al., 2003). Finally, amylases and DBEs may also be responsive to changes in stromal pH, which cycles between pH 8 during the day versus pH 7 during the night (Beck and Ziegler, 1989).

Rather than controlling the timing of starch degradation, an alternative model has been proposed that transient starch is degraded continuously throughout the diurnal cycle, but a net synthesis occurs during the day (Preiss, 1982). This does not appear to be the case in Arabidopsis at least, because Zeeman et al. (2002b) showed there was no turnover of granular starch during the day and that degradation commenced only during the dark portion of the diurnal cycle. Thus, starch degradation appears to be controlled at both the level of gene expression and by the subtle responses of the enzymes to various cellular conditions.

Structure of Starch Metabolizing Enzymes

Many enzymes that participate in starch metabolism share common structural features, i.e. similar domains responsible for substrate binding and catalytic activity. The α-amylase superfamily of starch hydrolytic enzymes are those enzymes involved in the hydrolysis and/or transfer of α-glucan linkages (Jespersen et al., 1991; Jespersen et al., 1993). This family contains a characteristic $(\beta/\alpha)_8$ barrel, which forms a cylinder containing the active site of the enzyme. α-Amylases are the most well characterized members of this family, but SBE and DBE in particular, also possess the same structural features (MacGregor et al., 2001). Within the $(\beta/\alpha)_8$ barrel, three critical amino acids, one glutamic acid and two aspartic acid residues, are absolutely required for the catalytic activity of these proteins (MacGregor, 1996). Furthermore, two histidine residues have been demonstrated to be important for transition state stabilization. Additional studies into how the structural features

of starch metabolizing enzymes relate to catalysis and substrate specificity will provide insights into the *in vivo* substrates for these proteins, and how the proteins might interact with one another.

α-Amylase

The pathway of starch degradation commences with the initial attack of the starch granule structure. In germinating maize seeds, α-amylases are the first enzymes secreted by the scultellum and aleurone (Subbarao et al., 1998). Similarly in barley, α-amylase is synthesized immediately by the embryo at the onset of germination (MacGregor and Matsuo, 1982), consistent with the idea that this enzyme is critical for initiating the degradative process. In leaves, α-amylases are differentially regulated throughout the diurnal cycle, such that there is a two-fold increase in total activity at the onset of the dark period (Ghiena et al., 1993).

α-Amylase is the primary enzyme that has been demonstrated to effectively begin the degradation of insoluble starch (Beck and Ziegler, 1989). Thus, this enzyme has the unique ability to cleave linkages in crystalline substrates. Once an initial solubilization of the starch is accomplished, several enzymes are capable of acting on the resulting oligosaccharides, both branched and linear dextrins. Consequently, other degradative enzymes primarily act on the products of α-amylolysis.

α-Amylase is an endo-acting enzyme, meaning that it hydrolyzes internal α(1→4) linkages in glucose polymers, usually releasing glucose polymers larger than maltotriose (MacGregor et al., 2001). The importance of this enzyme to efficient starch degradation has been confirmed by genetic analysis. The Arabidopsis *sex4* mutant is deficient in a specific

isoform of α-amylase (Zeeman et al., 1998a). Mutant plants are impaired in their capacity to degrade starch, and thus accumulate three-fold more starch than the wild-type. The excess starch accrues gradually as the plants age, owing to the incomplete degradation at the end of each diurnal cycle. Even though residual α-amylolytic activity remains in *sex4* mutant plants, it is clear that these activities cannot compensate for the loss of the *sex4* gene product. So, like most of the classes of starch biosynthetic enzymes, specific isoforms of α-amylase may have unique functions that cannot be fully substituted for by other isoforms.

β-amylase

β-amylases comprise a second class of hydrolytic enzymes. In contrast to α-amylases, β-amylases are exo-acting enzymes, releasing maltose from the non-reducing end of a glucose polymer. In Arabidopsis β-amylase accounts for over 80% of the amylolytic activity of the chloroplast (Trethewey and Smith, 2000). Other β-amylase activities are extra-chloroplastic, but the function of β-amylases localized in the cytosol has not been determined. In barley and wheat, β-amylase is synthesized during endosperm development and stored in a latent form, and are activated upon germination by endopeptidases (Guerin et al., 1992). Maize and rice β-amylase are also synthesized *de novo* by the aleurone layer (Wang et al., 1997).

The specific role of β-amylase during germination was investigated in soybean (Hildebrand and Hymowitz, 1981). The authors found that soybean lines with undetectable seed β-amylase activity are unaffected in the starch metabolism of the developing or germinating seed. They speculate that β-amylase has no essential role in mobilizing

carbohydrate during germination of soybean. Similarly, lines of rye that are deficient in β-amylase activity are fully capable of germinating normally (Daussant et al., 1981).

Ascertaining the role of β-amylase in transient starch degradation has been equally elusive. Characterization of the Arabidopsis *ram1* mutant, which shows a significant reduction in total β-amylase activity, indicates no defect in either starch synthesis or degradation (Laby et al., 2001). Plants grow normally and are generally not impaired in carbohydrate metabolism. In contrast, expression of an antisense β-amylase form in potato results in a starch excess phenotype in which plants were reduced by 20-40% in their capacity to degrade transitory leaf starch (Scheidig et al., 2002). Together, these results suggest that some isoforms of β-amylases may be critical for starch degradation, but others may be substituted for by one or more other degradative enzymes, including other forms of β-amylase.

α-Glucosidase

The primary function of α-glucosidase (maltase) is the hydrolysis of maltose to glucose. Data show that this enzyme is also capable of releasing glucose from some longer oligosaccharides up to maltoheptaose (Sun et al., 1995). The α-glucosidase of barley endosperm and pea chloroplast has been shown to attack native starch granules (Sun and Henson, 1990; Sun et al., 1995). These data implicate this enzyme, together with α-amylase, in making the granule more accessible to other degradative enzymes, e.g. DBE or β-amylase.

There is also some evidence that α-glucosidase has a wide range of substrate specificities. Sun et al. (1995) purified α-glucosidase from pea leaves and found that the enzyme was capable of hydrolyzing α(1→2), α(1→3), and α(1→6) linkages in addition to

the standard α(1→4)-type. While it is not clear if such linkages contribute to the composition of the starch granule, α-glucosidase would have the capacity to cleave such linkages, where other degradative enzymes would not. Thus, α-glucosidase may serve a specific function in the degradation of these linkages types that could not be substituted by other enzymes.

A significant synergism has also been demonstrated between the α-glucosidase and α-amylase of barley. Granules that were incubated with a combination of α-glucosidase and α-amylase were degraded at a rate 8-11 times faster than the sum of the rates of α-glucosidase and α-amylase acting independently. Hence, like other enzymes of starch synthesis and degradation, these two proteins may also work in concert to efficiently metabolize glucose polymers.

Debranching Enzyme

Of the proteins involved in starch degradation, DBEs are the major enzymes capable of efficiently hydrolyzing α(1→6) linkages. Thus, DBE activity is required regardless of whether the phosphoryolsis or hydrolytic pathways are operating. Cleaving of α(1→6) linkages in dextrins would make the products more accessible to nearly all other enzymes of starch degradation.

Similar to the system described above for α-amylase and α-glucosidase, a particular synergism is predicted to exist between β-amylase and the DBEs. Jiahua (1999) studied the kinetics of starch degradation when hydrolysis is accomplished by combinations of β-amylase and pullulanase. The data indicate that debranching of substrates removes the α(1→6) linkages that would block continued action by β-amylase. DBE does not increase

the overall number of chains on which β-amylase can act, but rather, the substrates have a more effective chain length for action by β-amylase (Jiahua, 1999). Debranching enzymes will be discussed in further detail in a following section.

Disproportionating Enzyme

D-enzyme essentially combines short maltooligosaccharides (MOS) into longer α-glucan chains, thereby making them better substrates for α-amylase, β-amylase, and starch phosphorylase during the process of starch degradation (Figure 1.3D). Studies in *Chlamydomonas* have suggested that D-enzyme performs a function in amylopectin synthesis, but equivalent studies of the Arabidopsis D-enzyme have suggested that this enzyme performs a role only in degradation (see previous section). In the Arabidopsis *dpe1* mutant, plants are impaired in their ability to degrade starch and accumulate 8-fold more MOS during the dark (Critchley et al., 2001). The MOS apparently cannot be effectively metabolized by other enzymes, suggesting a critical function for D-enzyme in starch degradation. These data are consistent with the model that D-enzyme recycles the MOS into bigger chains, which are better metabolized by α-amylase and/or starch phosphorylase. Furthermore, the fact that the overall rate of starch degradation in *dpe1* is significantly reduced suggests that the increased levels of MOS affect an enzyme that attacks the granule directly, or D-enzyme itself is able to act on crystalline starch (Critchley et al., 2001).

Starch Phosphorylase

Starch phosphorylase catalyzes the reversible phosphorolysis of an α-glucan chain from the non-reducing end, releasing Glc-1-P. For an oligosaccharide to be used as a substrate by starch phosphorylase, it must be longer than maltotetraose. The evidence for the

functioning of starch phosphorylase in starch degradation comes from a study of pea chloroplast phosphorylase. Kruger and ap Rees (1983) observed that starch phosphorylase releases Glc-1-P from ^{14}C-labeled starch granules. The authors speculate that during the night, P_i concentrations and pH would favor the degradative direction of the phosphorylase activity, versus the biosynthetic reaction. However, the precise role for the enzyme in either starch biosynthesis or degradation has not been conclusively ascertained, either by genetic analysis or biochemical characterization.

R1 Protein

The phosphorylation level of starch has been shown to be an important factor in starch degradation. Specifically, in plant strains where there is reduced phosphorylation of starch, degradation is impaired. The R1 protein has been shown to be an important factor in determining the phosphorylation level of starch (Lorbeth et al., 1998). Antisense expression of R1 in potato plants causes an overall reduction in the phosphate content of the starch. Furthermore, leaves of these plants fail to degrade starch, even after prolonged periods of darkness. Cold-sweetening, a conversion of starch to sucrose that occurs during storage, does not occur in plants with reduced R1 protein. Thus, the apparent defect is that of lower phosphate starch, which leads to a reduced capacity to degrade starch.

Similar to the antisense potato lines, Arabidopsis plants with missing or altered R1 protein are impaired in starch degradation. Plants homozygous for the Arabidopsis mutant *sex1* retain a high starch content even after prolonged periods of darkness (Caspar et al., 1991). Cloning of the *sex1* gene identified it as coding for the R1 protein (Yu et al., 2001). The enzymatic activity of R1/SEX1 is that of an α-glucan dikinase, directly implicating this

protein as controlling the phosphorylation of starch (Ritte et al., 2002). The authors speculate that phosphorylation of starch results in increased hydrophilicity, which would make glucan chains more accessible to starch-degradative enzymes.

Transport of Degradation Products

In storage organs, e.g. cereal endosperm, starch degradation is largely extracellular. The primary degradation product is Glc, which is absorbed by the scutellum, converted to Suc, and used by the embryo during germination (Beck and Ziegler, 1989). However, in leaf tissue, compartmentation requires that degradation products be exported from the chloroplast. Since phosphorolysis is speculated to play a much greater role in leaf starch catabolism, the degradation products include both Glc and Glc-1-P. Most studies of metabolite flux in the leaf have shown that Glc-1-P is converted to triose phosphate, which is then exported from the plastid. Hexose phosphates are then generated from the triose phosphate in the cytosol. In mutants where there is a defect in the TPT, there is still an ability to degrade starch. This supports the hypothesis that the amylolytic pathway is capable of producing Glc and this product is able to be exported from the plastid. A transporter capable of exporting Glc directly has been identified in chloroplast membranes (Weber et al., 2000). This protein has the capacity to transport Glc at rates comparable to those of the overall process of starch degradation, but the rate is dependent on a steep concentration gradient between the stroma and cytosol (Weber et al., 2000). During the dark, such a gradient would likely exist, implicating this transporter as the primary shunt for glucose to reach the cytosol.

Function of Starch Debranching Enzymes

Numerous studies indicate that starch debranching enzymes (DBEs) may perform a dual function in starch metabolism, in the synthesis and degradation of amylopectin. This section will review the types of starch debranching enzymes and the currently available evidence for this proposed dual function.

Types of Starch Debranching Enzymes

In plants and bacteria, there are two types of α(1→6) glucan hydrolase, isoamylase- and pullulanase-type DBE. These are classified based upon their *in vitro* substrate specificity and amino acid sequence similarity to defined bacterial enzymes. Proteins of both classes perform the same enzymatic reaction, i.e. hydrolysis of α(1→6) glucosyl bonds, but differ in the substrates that they prefer. Specifically, isoamylase-type DBEs cleave α(1→6) linkages in amylopectin and glycogen, but are not active toward the isomaltotriose polymer pullulan (Manners, 1971). Pullulanase-type DBEs, on the other hand, prefer to cleave linkages in pullulan and limit dextrins, but are not active or only slightly active towards glycogen and amylopectin (Manners et al., 1970; Manners, 1971). Doehlert and Knutson (1991) observed that pullulanase prefers to hydrolyze exposed branch linkages, where isoamylase hydrolyzes less-exposed linkages. Thus, the differences in specificity are fundamentally related to the length of the branches as well as the spaces between subsequent branches.

Evolutionary Conservation

Both classes of enzymes have been conserved separately throughout the evolution of plants and bacteria (Beatty et al., 1999). Like other members of the α-amylase superfamily

of starch hydrolytic enzymes, isoamylase- and pullulanase-type DBEs possess the $(\beta/\alpha)_8$ catalytic domain (Jespersen et al., 1991; Jespersen et al., 1993). Additional motifs are also conserved among members of either the isoamylase or pullulanase classes (Beatty et al., 1999). Phylogenetic studies indicate that isoamylases and pullulanases diverged from one another prior to the divergence of plants from bacteria (Beatty et al., 1999). Thus, each isoform must perform a specific function that cannot be fully compensated for by DBEs of the other class. This specificity presumably provided the basis for selection of each isoform independently through evolution.

The isoamylase-type DBE isoform *Su1* was recently suggested to be under selection during the domestication of maize (Whitt et al., 2002). When comparing the sequences of 30 maize lines to teosinte, the *Su1* gene appears to have very low sequence diversity. Since maize generally possesses a great deal of genetic diversity, those genes with less sequence heterogeneity may represent targets of selection. Loci that have levels of diversity similar to *Su1* include other genes in the starch pathway and those relating to plant morphology, e.g. *teosinte branched* (*tb1*). Presumably, Native Americans selected for, among other things, traits relating to the use of maize as a food crop. Enzymes of the starch biosynthesis pathway that are most efficient would no doubt be selected to meet this need.

Protein Characteristics of DBEs

Isoamylase-type DBEs are speculated to function in a protein complex. Evidence from potato tubers and rice endosperm suggests that the native form of isoamylase forms a large multimeric complex. In rice, the isoamylase complex was demonstrated to be a homomultimer of 340 - 490 kDa, i.e. approximately 4-6 polypeptides (Ishizaki et al., 1983;

Fujita et al., 1999). In maize, recombinant SU1 expressed in *E. coli* exhibits hydrolytic activity, supporting the hypothesis that no other plant factors are required for activity, and not inconsistent with the idea that this protein assembles as homomultimer (Rahman et al., 1998).

However, recent biochemical studies of the potato isoamylase have shown that two separate polypeptide forms, StISA1 and StISA2, form a heteromultimeric complex (Hussain et al., 2003). Evidence to support this hypothesis is provided by analysis of the native enzyme isolated from potato tubers. The components of the high molecular weight complex (450-500 kDa) were shown to be StISA1 and StISA2 on the basis of isoform-specific antibodies. Experiments also showed that StISA1 and StISA2 work synergistically in the degradation of certain substrates. The combination of StISA1 and StISA2 showed 81% more activity on β-limit dextrin than would be expected from the sum of individual *Stisa1* and *Stisa2* activities. Since StISA2 is not active by itself, StISA1 may be required for catalytic activity of StISA2, or else StISA2 stimulates StISA1 to hydrolyze certain substrates. The authors propose that this might be similar to the interaction of the two subunits of AGPase, in which one subunit is primarily catalytic and the other subunit is primarily regulatory, even though both subunits possess structural features consistent with a role in catalysis (Hussain et al., 2003).

Studies in *Chlamydomonas* further support the importance of a multimeric protein complex in the function of isoamylase. Mutations in the *sta7* locus cause complete loss of detectable isoamylase activity, which results in the accumulation of phytoglycogen at the expense of normal amylopectin (Dauvillee et al., 2001a). The *sta8* mutant causes a 35% reduction in isoamylase activity and also accumulates phytoglycogen along with some

altered amylopectin. In gene dosage experiments where two *sta7* alleles were placed in combination with one wild-type allele, there was also approximately a 35% reduction in isoamylase activity, but no phytoglycogen accumulated (Dauvillee et al., 2001a). This experiment showed that the total amount of isoamylase activity was not the reason for the accumulation of phytoglycogen in the *sta8* mutant, but rather, the loss of some other factor, such as a structural component in the isoamylase protein complex. This hypothesis is supported by analysis of the molecular mass of the native enzyme, which was reduced by one-half in *sta8* compared to wild-type (Dauvillee et al., 2001b). Despite the reduction in size, the mutant enzyme retained catalytic activity as measured *in vitro*. Thus, *STA8* may represent another form of isoamylase, serving a regulatory function *in vivo*, analogous to the complex described in potato. Alternatively, *STA8* may not be an isoamylase, but rather, another type of structural protein involved in the isoamylase complex formation with *STA7*.

Biochemical evidence shows pullulanase-type DBE acts as a monomer. However, an interesting feature of the pullulanase-type DBE from maize and spinach are the apparent isomeric polypeptides, which show different mobilities on isoelectric focusing (IEF) gels. Detailed characterization of the spinach pullulanase showed that a polypeptide, encoded for by a single gene, migrates as seven different forms on IEF gels (Henker et al., 1998; Renz et al., 1998). These different forms corresponded to differently oxidized or reduced states, which represented less or more active forms, respectively. Similarly, the maize pullulanase ZPU1 migrates as a doublet on SDS-PAGE of 100 and 105 kD (Beatty et al., 1999). The smaller form is considered to be catalytically active, and the larger form to be inactive.

Involvement of DBEs in Starch Degradation

The hydrolytic activity of DBEs points toward a *prima facie* role in starch degradation. Effective mobilization of starch when it is required by the plant would necessitate the cleavage of α(1→6) linkages. Of the enzymes involved in starch degradation, i.e. amylases, starch phosphorylase, and α-glucosidase, only DBEs would efficiently cleave these linkages. As described above, DBEs work synergistically with other degradative enzymes, especially β-amylase.

During the germination of barley, the role of the pullulanase-type DBE has been described most extensively. This is due largely to the importance of barley degradative enzymes during the malting of beer. Pullulanase-type DBE in barley is synthesized *de novo* during germination and stored during endosperm development in an inactive form conjugated to a limit dextrinase (LD) inhibitor. Cysteine proteases released from the aleurone layer activate the enzyme by releasing the LD inhibitor, generating an active protein. Thus, pullulanase-type DBE in barley can exist in both free and bound forms. In maize, there is no evidence for a LD inhibitor, so the mechanism for activation of the stored form upon germination is not understood. Also unclear is the mechanism by which pullulanase is secreted from the aleurone layer. In both maize and barley, pullulanase contains sequences for targeting to the plastid (Beatty et al., 1999; Burton et al., 1999), and not a signal peptide for targeting to the secretory pathway via the ER. Burton et al. (1999) argued that pullulanase was not secreted from the aleurone until the final stages of germination when these cells burst, although no direct evidence for this hypothesis is available. The precise role of the isoamylase-type DBE in starch degradation remains to be discerned.

Involvement of DBEs in Starch Synthesis

The first evidence for the role of DBEs in starch synthesis came from studies of the *sugary1* (*su1*) mutant of maize (Correns, 1901). *su1*- mutants are recessive mutations which condition an altered kernel phenotype when homozygous. At maturity, kernels appear shrunken and translucent, owing to changes in the carbohydrate composition of the endosperm. Specifically, the kernels accumulate a water soluble polysaccharide, which exhibits a similar structure and branching pattern to bacterial and animal glycogen, hence it is called phytoglycogen (Sumner and Somers, 1944). Phytoglycogen has approximately twice the branch frequency of amylopectin, with branches more randomly spaced, rather than clustered (Shannon and Garwood, 1984). There is a concomitant decrease in the amount of granular starch. *su1* kernels also accumulate soluble sugars, especially sucrose, which has made sugary maize a traditional sweet corn variety since pre-Columbian times (Garwood and Vanderslice, 1981).

Pan and Nelson (1984) reported that the enzymatic deficiency in *su1*- endosperm was in a DBE. They found a tight correlation between the number of doses of wild-type alleles with amount of residual DBE activity in the endosperm. Chromatographic separation of DBE activities revealed the loss of one activity peak and reduction of other peaks in *su1* extracts compared the wild-type. The DBE activity that Pan and Nelson (1984) identified as missing from *su1*- mutants had activity toward pullulan, and would therefore be classified as a pullulanase.

Cloning of the *Su1* gene confirmed the molecular defect as that of a DBE (James et al., 1995), however the predicted polypeptide had strong sequence identity with bacterial isoamylases, not pullulanases, as would be predicted from Pan and Nelson's

characterization. The apparent inconsistency was resolved upon identification of a non-linked gene coding for the pullulanase-type DBE isoform, *Zpu1* (Beatty et al., 1999). It was shown that, in fact, the ZPU1 polypeptide is altered in *su1* mutants, and the activity is greatly reduced. Further confirmation was provided by expression of recombinant SU1 in *E. coli*, which demonstrated that this enzyme could, on the basis of its substrate specificity, be classified as an isoamylase-type DBE. Thus, the defect in the isoamylase-type DBE SU1 causes a reduction in the activity of the pullulanase-type DBE ZPU1 as a secondary, pleiotropic effect.

Analogous isoamylase mutations in other species have similar effects on carbohydrate composition. Rice *su1* mutants also accumulate phytoglycogen at the expense of normal starch. Like the maize mutant, there is a decrease in pullulanase activity, even though the primary enzymatic defect is that of the isoamylase-type DBE. Rice *su1* mutants accumulate phytoglycogen in the central part of the endosperm and starch near the periphery (Nakamura et al., 1997). The extent to which the starch region extended inward varied with respect to the particular *su1* allele examined. Nakamura and colleagues correlated the starch containing regions with areas that possessed higher levels of pullulanase activity (Nakamura et al., 1997; Kubo et al., 1999). From these data, the authors speculate that the pullulanase is essential in compensating for the loss of the isoamylase in producing the residual starch in *sugary* mutant rice grains.

Isoamylase mutants in *Chlamydomonas* (Mouille et al., 1996; Dauvillee et al., 2001a), Arabidopsis (Zeeman et al., 1998b), and barley (Burton et al., 2002b) exhibit similar phenotypes to *sugary* mutant maize and rice, i.e. phytoglycogen accumulation and reduction in starch content. In contrast, these species show no reduction in pullulanase-type DBE

activity, suggesting that loss of isoamylase is the primary determinant of the phytoglycogen-accumulating phenotype, and pullulanase is not important in this regard.

The nature of the pleiotropic interaction between pullulanase and isoamylase was investigated by Wu et al. (2002). This study observed that like *su1* mutants, the maize *sh2* mutants exhibit reduced activity of the pullulanase-type DBE compared to the wild-type. *sh2* and *su1* mutants are similar in that they both accumulate soluble sugars, especially sucrose. Enzyme inhibition resulting from increased cellular sucrose concentration was proposed to be the reason for the reduced activity of ZPU1 (Wu et al., 2002). This control may be mediated by redox signaling, since, like the barley enzyme (Cho et al., 1999), maize ZPU1 can be activated by reduced by thioredoxin. The conclusion drawn from this study is that the reduction in ZPU1 activity in *su1* mutants is the result of post-translational redox modification brought about by the increased cellular sucrose concentrations.

Models Describing the Function of DBEs in Starch Synthesis

Among the first attempts to describe the cause of the phytoglycogen-accumulating phenotype, Erlander (1958) proposed that phytoglycogen was an intermediate in the starch pathway, and that DBE acted upon this intermediate to remove some of the branches, yielding amylopectin (Figure 1.5A). However, Manners and Rowe (1968) found that the particular branching pattern in amylopectin cannot be formed by debranching of a glycogen precursor. The molecular weight of phytoglycogen is also much smaller than amylopectin, suggesting that debranching of this type of precursor could not directly yield amylopectin.

The glucan trimming model (Figure 1.5B) proposes that DBEs are directly involved in starch biosynthesis, by selectively removing branches from an amylopectin precursor

polymer (Ball et al., 1996; Myers et al., 2000). In contrast to Erlander's model, glucan trimming would be described as a continuous process involving a certain degree of competition between SBE and DBE. This model suggests that SBE introduces α(1→6) linkages, but does so in a random manner, such that there may be more branches introduced than would be possible for crystallization. If these branches are not removed, they would

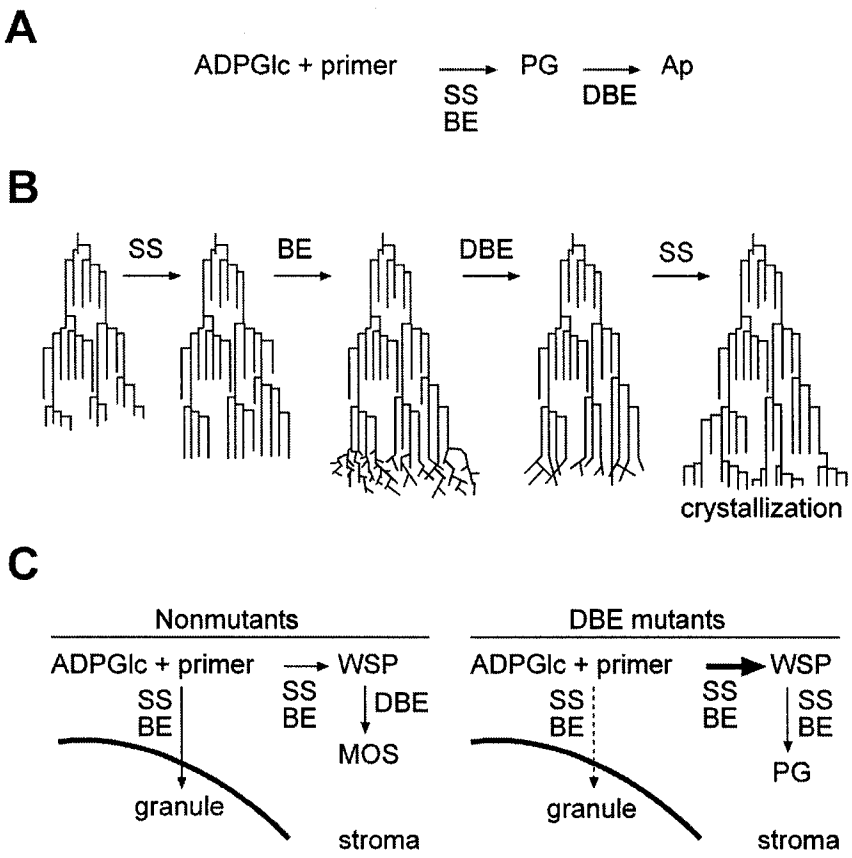

Figure 1.5. Models of DBE function in starch synthesis. **A.** Phytoglycogen intermediate model proposes phytoglycogen is an intermediate in amylopectin biosynthesis. **B.** Glucan trimming model proposes DBEs function on an amylopectin precursor, facilitating crystallization. **C.** WSP clearing model proposes DBEs act in stroma to prevent accumulation of soluble glucan. Modified from Myers et al. (2000).

continue to be acted upon by SS and SBE to the point where the entire nascent chain would fail to crystallize, and thus be destined to remain soluble, leading to phytoglycogen accumulation. The action of DBEs to promote crystallization would be favored entropically, since when glucan chains are soluble, water is forced to be ordered around them.

This model implies that DBEs are acting directly on an amylopectin precursor polymer. Changes in DBE activity, therefore, might be expected to alter the structure of the amylopectin, since one of the activities important for determining branch size and number is missing or altered. Such alterations in chain length distribution in the residual endosperm starch of sugary mutant rice have been observed (Nakamura et al., 1996; Nakamura et al., 1997). Namely, there is an increase in short A chains, relative to the wild-type amylopectin chain length distribution, suggesting that in the absence of DBE, these chains fail to become removed prior to crystallization.

An alternative model to explain the accumulation of phytoglycogen in DBE mutants is the water soluble polysaccharide (WSP) clearing model (Zeeman et al., 1998b) (Figure 1.5C). This proposes that SS and SBE may act separate from the starch granule to elongate and branch MOS. DBEs normally prevent accumulation of this material by degrading it before it reaches the size and/or quantity of phytoglycogen. In the absence of DBEs, ADP-Glc would be diverted from productive starch synthesis occurring on the surface of the granule to those reactions occurring in the stroma. This model implies an indirect function, because the DBEs would not be involved in directly determining the structure of amylopectin. The evidence to support this model comes from study of the Arabidopsis *dbe1* mutant, which is defective in isoamylase-type DBE. Starch in *dbe1* leaves exhibits an

apparently normal distribution of linear chain lengths, which would not be expected if DBE is required for structural determination.

A variation of the WSP clearing model was proposed by Burton et al. (2002b). This hypothesis proposes that DBEs are required for proper starch granule intiation. In addition to causing phytoglycogen production, the barley *isa1* mutant results in a significant increase in the number of starch granules per amyloplast. The authors suggest that during early starch formation, soluble glucan may spontaneously crystallize, thereby initating the starch granule. The function of DBE is to reduce the concentration of the soluble glucan, thereby decreasing the frequency of these nucleation events. If the concentration of soluble glucan remains high, more frequent initiations would occur. Phytoglycogen accumulation might be the result of uncontrolled initiation brought about by failure of DBE to properly remove soluble glucan from the stroma. Alternatively, the authors propose isoamylase might cleave glucose from a glucosylated protein primer, preventing the initiation of amylopectin and phytoglycogen molecules.

Evidence supporting the glucan trimming model is provided by studies of starch synthesis in Arabidopsis. Nielsen et al. (2002) used a short $^{14}CO_2$ pulse labeling technique to observe the types of glucan chains being created at the surface of the starch granule. They observed that a population of chains exists transiently that are not present in the mature starch. Specifically, there are more chains in the precursor polymer than go on to form crystalline amylopectin. While the fate of these chains is not clear and no direct evidence is currently available, it is possible that these chains are removed by DBE. The reason that the residual starch of the Arabidopsis *dbe1* mutant did not have altered amylopectin chain length distribution is also not clear. Perhaps the function in trimming is substituted by another

isoform of isoamylase or pullulanase, but this form of DBE apparently does not prevent the accumulation of phytoglycogen. Currently available data do not irrefutably support one model over another, and the possibility remains that DBEs are performing multiple functions. The role of DBEs described by one model is not necessarily exclusive of the functions described by the other models.

Experimental Approach

Similar to many of the studies described in this review, the research presented in this dissertation takes a genetic approach to investigate the functions of DBE genes during the synthesis and degradation of starch in the maize plant. In a broad sense, genetic studies seek to identify strains that possess heritable differences (i.e. mutants) in a particular aspect of their physiology or development, and then describe the molecular and/or biochemical defect that gives rise to such differences. Such studies often provide insights into the mechanism of the physiological or developmental process in the non-mutant condition. Hence, through the characterization of DBE mutants, I intend to characterize the function of these enzymes.

Two DBE genes have been identified in maize, and the first objective of this project is to identify additional genes with new or redundant functions. Once the entire complement of those factors involved in the debranching enzyme activity within the plant is recognized, the relative contribution of each gene can be resolved. Specifically, the second objective will be to evaluate the function of each DBE isoform through characterization of expression, activity, and influences on other starch metabolizing enzymes. A possible role of each gene in starch degradation will be contrasted to its contribution to starch biosynthesis. A genetic and molecular evaluation of the function of each of the DBE genes both independently and

in combination with mutant alleles of other starch biosynthetic genes will begin to address the importance of these enzymes to the carbon metabolism of the maize plant.

Dissertation Organization

This dissertation includes four papers of which I am the primary author. Chapter 1 includes a review of the relevant literature and the experimental approach. Chapter 2 is a paper that was published in the journal *Plant Physiology*. Chapter 3 is a paper that was published in the journal *The Plant Cell*. Chapter 4 is a paper published in the *Journal of Applied Glycoscience,* which supplements the data presented in Chapter 3. Chapter 5 is a paper to be submitted for publication in the journal *Plant Molecular Biology*. Chapter 6 is a general concluding chapter, where the findings of the four papers are summarized. An appendix describing the identification of maize *ZmIso2* and *ZmIso3* mutants by reverse genetics is also included.

All of the papers were written by me with the guidance and assistance from my co-major professors, Dr. Alan Myers and Dr. Martha James. The first two papers are multi-author papers. The analysis of carbohydrate composition (Chapter 2) and amylopectin chain length distribution (Chapter 2 and Chapter 3) were conducted by Dr. Christophe Colleoni. I conducted all other experiments described in this dissertation.

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CHAPTER 2. MOLECULAR STRUCTURE OF THREE MUTATIONS AT THE MAIZE *su1* LOCUS AND THEIR ALLELE-SPECIFIC PHENOTYPIC EFFECTS

A paper published in the journal *Plant Physiology* [1]

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Abstract

Starch production in all plants examined is altered by mutations of isoamylase-type starch debranching enzymes (DBE), although how these proteins affect glucan polymer assembly is not understood. Various allelic mutations in the maize (*Zea mays* L.) gene *sugary1* (*su1*), which codes for an isoamylase-type DBE, condition distinct kernel phenotypes. This study characterized the recessive mutations *su1-Ref*, *su1-R4582::Mu1*, and *su1-st*, regarding their molecular basis, chemical phenotypes, and effects on starch metabolizing enzymes. The *su1-Ref* allele results in two specific amino acid substitutions without affecting the *Su1* mRNA level. The *su1-R4582::Mu1* mutation is a null allele that abolishes transcript accumulation. The *su1-st* mutation results from insertion of a novel transposon-like sequence, designated *Toad*, which causes alternative pre-mRNA splicing. Three *su1-st* mutant transcripts are produced, one that is nonfunctional, and two that code for modified SU1 polypeptides. The *su1-st* mutation is dominant to the null allele *su1-R4582::Mu1*, but recessive to *su1-Ref*, suggestive of complex effects involving

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quaternary structure of the SU1 enzyme. All three *su1-* alleles severely reduce or eliminate isoamylase-type DBE activity, although *su1-st* kernels accumulate less phytoglycogen and sucrose than *su1-Ref* or *su1-R4582::Mu1* mutants. The chain length distribution of residual amylopectin is significantly altered by *su1-Ref* and *su1-R4582::Mu1*, whereas *su1-st* has modest effects. These results, together with *su1* allele-specific effects on other starch metabolizing enzymes detected in zymograms, suggest that total DBE catalytic activity is not the sole determinant of *Su1* function, and that specific interactions between SU1 and other components of the starch biosynthetic system are required.

Introduction

Much of the metabolic activity in plants is directed toward production of starch from reduced carbon formed during photosynthesis. Starch that accumulates in leaves during the light phase of a diurnal cycle is degraded in the dark phase. Sucrose is transported from this source tissue to non-photosynthetic storage tissues, where it supplies glucose units for the synthesis of starch that serves as the carbon reserve for the subsequent generation. Starch biosynthesis is highly conserved throughout the plant kingdom, presumably owing to this central role in plant physiology and reproduction. Nevertheless, there is little understanding of the molecular mechanisms that determine how the precise structure of starch is achieved in either leaves or storage tissues.

Starch comprises two homopolymers of glucose, amylopectin and amylose, and it is the ordered architectural structure of amylopectin, the most abundant component, that confers crystallinity to the starch granule (Imberty et al., 1991; Gallant et al., 1997). Amylopectin synthesis involves the combined actions of multiple isoforms of starch

synthases (SS), which elongate linear chains via the introduction of α-(1→4) glycosidic bonds, and starch branching enzymes (BE), which catalyze the formation of α(1→6) branch linkages (for reviews see Smith, 1999; Kossman and Lloyd, 2000; Myers et al., 2000). The non-random placement of branch linkages and determination of chain length results in a complex, hierarchical structure that renders the molecule both crystalline and insoluble.

A broad body of genetic evidence in several plant species indicates that α(1→6) glucan hydrolases, i.e., starch debranching enzymes (DBE), are necessary for the normal formation of crystalline starch granules. Two types of DBEs have been identified in plants, distinguished by their substrate specificities (Doehlert and Knutson, 1991; Lee and Whelan, 1971). Isoamylase-type DBEs cleave α(1→6) branch linkages in amylopectin and glycogen, but do not hydrolyze the chemically identical bonds in pullulan, an α(1→6)-linked maltotriose polymer. In contrast, pullulanase-type DBEs, also referred to as R-enzymes or limit-dextrinases (Manners, 1997), hydrolyze α(1→6) linkages of pullulan and to a lesser degree amylopectin, but have little or no activity toward glycogen. Sequence comparisons among plant and bacterial α(1→6) glucan hydrolases indicate that the pullulanase-type and isoamylase-type DBEs have been conserved separately in evolution (Beatty et al., 1999).

The first genetic evidence for DBE involvement in starch biosynthesis came from mutations of the maize *sugary1* (*su1*) gene, described a century ago in the scientific literature (Correns, 1901). These mutations result in the accumulation of soluble sugars and a water-soluble polysaccharide (WSP) termed phytoglycogen in the kernel (Morris and Morris, 1939). In *su1* kernels, the reduction in amylopectin content approximately matches the abundance of phytoglycogen, suggesting a diversion from biosynthesis of the normal insoluble branched glucan to the water-soluble form. Similar to glycogen, phytoglycogen

has approximately twice the frequency of branch linkages as amylopectin, a shorter average chain length, and a more uniform chain length distribution (Yun and Matheson, 1993). More recently, the accumulation of phytoglycogen also has been reported in mutants of rice, Arabidopsis, and *Chlamydomonas* (Mouille et al., 1996; Nakamura et al., 1996; Zeeman et al., 1998; Kubo et al., 1999). In each species, phytoglycogen accumulation correlates with a lack of DBE activity of the isoamylase-type (Mouille et al., 1996; Rahman et al., 1998; Zeeman et al., 1998; Beatty et al., 1999; Kubo et al., 1999). In maize and Arabidopsis the mutation responsible for this specific phenotype has been shown directly to reside in a gene that codes for an isoamylase-type DBE (James et al., 1995; Zeeman et al., 1998), and indirect evidence indicates this is likely to be the case in *Chlamydomonas* and rice.

The first described *su1* mutation (Correns, 1901) is now termed *su1-Ref.* As the allele name indicates, this mutation has served as the reference standard for most analyses of the phenotypic effects. Kernels homozygous for *su1-Ref* have a glassy, translucent, and shrunken appearance at maturity resulting from their altered carbohydrate composition. The total amount of phytoglycogen reported to be present in *su1-Ref* kernels has ranged from 25% - 35% of the total glucan (Creech, 1968; Shannon and Garwood, 1984). Several early reports indicated that *su1-Ref* kernels contained an altered BE activity, prompting speculation that phytoglycogen accumulation results from an alteration in the formation of branch linkages (Lavintman, 1966; Manners et al., 1968; Hodges et al., 1969; Tomalsky and Krisman, 1987). Previous analysis of *su1-Ref* mutant kernels also revealed the deficiency of a DBE, although that activity was of the pullulanase-type (Pan and Nelson, 1984). More recently, cloning of the *Su1* gene revealed that it codes for a DBE of the isoamylase-type (James et al., 1995), and that *su1-Ref* kernels are deficient in both isoamylase- and

pullulanase-type DBE isoforms (Beatty et al., 1999). This dual effect on both DBEs is also observed in endosperm tissue of rice *su1* mutants (Nakamura et al., 1996). However, pullulanase-type DBE apparently is unaffected in Arabidopsis leaves or the unicellular algae *Chlamydomonas* when mutations affecting an isoamylase-type DBE are present (Zeeman et al., 1998; Dauvillee et al., 2000).

The complexity of the starch biosynthetic system, including the involvement of multiple isozymes of SS, BE, and DBE, so far has precluded biochemical reconstitution of an architecturally accurate synthetic system. Pleiotropic effects in most phytoglycogen-accumulating mutants also has complicated the analysis of the role of isoamylase-type DBEs in the starch biosynthetic process. In this study, we have begun to exploit the great deal of allelic diversity that is available for the maize *su1* locus, towards the aim of establishing correlations between mutational effects on the SU1 polypeptide, specific enzymatic alterations, and defined changes in the chemical structures of glucan polymers. Three *su1* alleles in near-isogenic lines were compared to the nonmutant allele *Su1*. Two of the mutant alleles, *su1-Ref* and the transposon-induced allele *su1-R4582::Mu1* (James et al., 1995), condition severe glassy, shrunken, and translucent kernel phenotypes. The third, *su1-starchy* (*su1-st*), results in a relatively mild phenotype in which translucency is typically limited to the kernel crown (Dahlstrom and Lonnquist, 1964). This research examined the dominance relationships among these *su1* alleles, the quantity of phytoglycogen and sugars that accumulate in the kernels, the chain length distributions of the polymers present, and the effects of each allele on the activities of DBEs and other starch metabolizing enzymes. The molecular nature of each mutation also was determined. In the course of this analysis, a novel transposon-like sequence, termed *Toad*, was identified at the *su1-st* locus. This

element was shown to affect the pre-mRNA splicing of the exon in which it resides. The three *su1-* mutations had diverse effects on the compositions and structures of kernel glucans, as well as on the activities of other starch metabolizing enzymes. However, direct correlation was not established between the apparent level of isoamylase-type DBE activity and the observed chemical phenotype. Together, the findings suggest that the effects of each mutation varied according to the primary structure of the SU1 polypeptide, and may relate to the assembly-state of the SU1 holoenzyme.

Results

Kernel morphologies and dominant/recessive relationships of the *su1-* mutations

Self-pollinated ears from *Su1/su1-Ref* or *Su1/su1-st* heterozygous plants contain progeny kernels segregating at the ratio of 1:3 for the mutant and nonmutant phenotypes. The typical glassy, shrunken, and translucent phenotype of *su1-Ref* homozygous kernels is evident (Fig 2.1A), and this is very similar to the kernel phenotype conditioned by *su1-R4582::Mu1* (James et al., 1995). The *su1-st* kernel phenotype is relatively slight (Fig. 2.1B) compared with that conditioned by *su1-Ref.* These mutant kernels are plump and starchy with translucency and wrinkling only on the kernel crown. Also, the *su1-st* mutation varies considerably in expressivity, with some kernels appearing near normal. Despite these distinct kernel phenotypes, *su1-st* is clearly identified as a mutation of the *su1* locus because it fails to complement *su1-Ref* and by definitive molecular characterization (see following section).

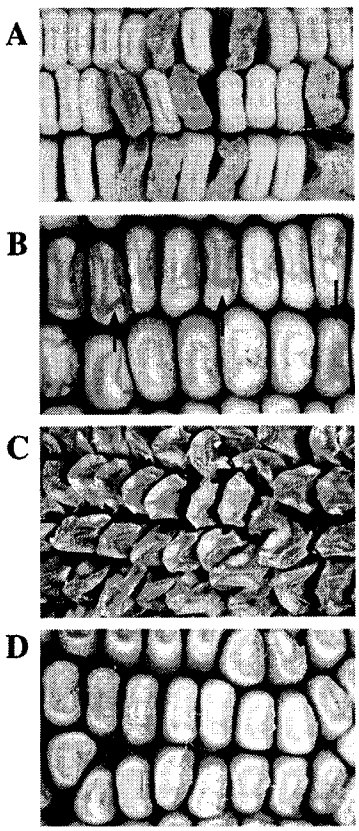

Figure 2.1. Kernel phenotypes of *su1*-mutants. **A.** The *su1-Ref* mutant phenotype. The ear shown was obtained by self-pollination of a *su1-Ref/Su1* heterozygote. **B.** The *su1-st* mutant phenotype. The ear shown was obtained by self-pollination of a *su1-st/Su1* heterozygote. Mutant kernels (examples indicated by the arrows) and wild-type kernels segregated at a frequency of approximately 1:3, respectively. **C.** Phenotype of *su1-Ref/su1-st* kernels. The ear shown is the result of a cross of a *su1-st/su1-st* plant as the male parent to a *su1-Ref/su1-Ref* plant as the female parent. **D.** Phenotype of *su1-R4582::Mu1/su1-st* kernels. The ear shown is the result of a cross of a *su1-R4582::Mu1/su1-R4582::Mu1* plant as the male parent to a *su1-st/su1-st* plant as the female parent.

Previous genetic analyses of the dominant/recessive relationship between *su1-st* and wild type (*Su1*) or *su1-Ref* alleles showed that the *su1-st* allele is recessive to both, although reciprocal crosses between *su1-Ref* and *su1-st* plants indicated subtle dosage effects of the *su1-st* allele (Dahlstrom and Lonnquist, 1964). This finding also was confirmed in this study, where progeny ears from reciprocal *su1-Ref* by *su1-st* crosses produced kernels that were uniformly shrunken, glassy, and translucent in appearance (Fig. 2.1C). Crosses also

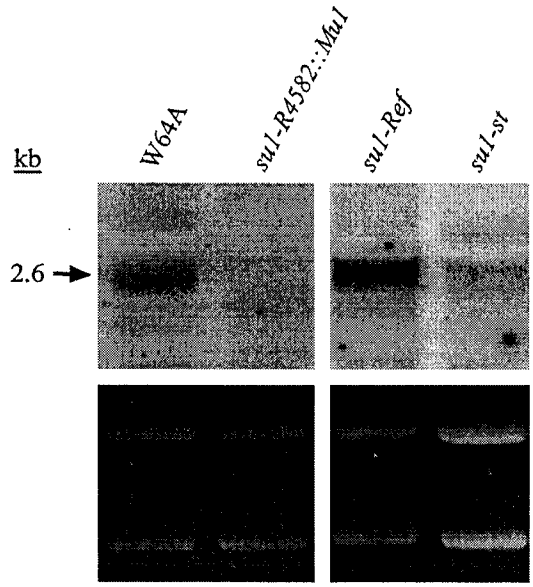

Figure 2.2. RNA gel blot analysis. Total RNA was extracted from endosperm of kernels harvested at 20 DAP, fractionated on a 1% agarose-formaldehyde gel, and hybridized with Su1 cDNA antisense RNA. RNA concentration variations can be visualized by the ethidium bromide-stained rRNA, shown in the lower panel. The lanes shown were run simultaneously in the same gel.

were made between *su1-st* plants and plants homozygous for *su1-R4582::Mu1*. The *su1-R4582::Mu1* allele is predicted to be a null allele, based on the presence of a *Mu1* transposable element in the first exon of the gene downstream of the translational start site (James et al., 1995). Progeny ears from the *su1-st* by *su1-R4582::Mu1* cross were uniformly like *su1-st* homozygotes in appearance, indicating that the *su1-st* allele is dominant to *su1-R4582::Mu1* (Fig. 2.1D). Given that *su1-Ref* and *su1-R4582::Mu1* condition nearly identical kernel phenotypes, the different dominance relationships of these two alleles with *su1-st* most likely reflect the presence or absence of a SU1 polypeptide.

Steady-state levels of Su1 mRNA in the various *su1*- mutants

Gene expression of *su1-Ref*, *su1-st*, and *su1-R4582::Mu1* relative to the nonmutant allele *Su1* was characterized in kernels at a mid-developmental stage by RNA gel blot

analysis. Total RNA was isolated from kernels harvested 20 DAP and hybridized with an antisense RNA probe from the 3' end of the Su1 cDNA. Kernels homozygous for *su1-Ref* produce transcripts of normal size that accumulate to wild type levels (Fig. 2.2). In contrast, kernels homozygous for *su1-R4582::Mu1* accumulated no detectable Su1 transcript. This supports the prediction that *su1-R4582::Mu1* is a null allele. Homozygous *su1-st* kernels at this stage accumulated a relatively slight amount of apparently normal-sized transcript. Thus, the partial function of the *su1* locus that is evident from the intermediate phenotype conditioned by *su1-st* (Fig. 2.1B) is provided by a relatively small number of transcripts.

The molecular nature of *su1-Ref*

Although the *su1-Ref* mutation has long been utilized for the production of traditional sweet corn varieties, the molecular basis for this mutation was not known. To characterize this allele, segments of *su1-Ref* kernel mRNA and genomic DNA were amplified by PCR, and their sequences were determined. Initially, all materials analyzed were obtained from the W64A genetic background. Sequence comparisons of *su1-Ref* to *Su1* revealed three nucleotide changes that would result in amino acid substitutions, a C to G transversion at nt 576, an A to G transition at nt 1100, and a T to C transition at nt 1819 (nucleotide and amino acid numbers refer to GenBank accession no. U18908). Translation would result in three amino acid changes relative to the wild type polypeptide SU1, phenylalanine to leucine at residue 163, glutamate to valine at residue 338, and tryptophan to arginine at residue 578. This mutant polypeptide is designated $SU1^{Ref}$. Similar analysis of commercial *su1-Ref* sweet corn varieties in the NK199 and Golden Cross Bantam backgrounds showed that only two of these nucleotide changes were present, specifically those resulting in the F163L and W578R

substitutions. These two mutations, therefore, most likely were present in the progenitor *su1-Ref* allele that has been used in existing commercial sweetcorn lines and introgressed into the inbred line W64A. The third mutation, E338V, most likely arose more recently and thus is not required for the phytoglycogen-accumulating phenotype conditioned by *su1-Ref.*

Sequence comparisons among α-(1→6) glucan hydrolases revealed numerous conserved sequence blocks, some of which are common to all members the α-amylase family (Jesperson et al., 1993) and some of which are specific to either the isoamylse-type or pullulanase-type DBE isoforms (Beatty et al., 1999). Two of the three substitutions in *su1-Ref* occur within conserved domains, specifically E338V in α-amylase region I, and W578R in the isomylase-specific domain IS-XII. The third mutation, F163L, lies just downstream of domain IS-VII but is not located in a conserved region of the enzyme. The only highly conserved residue affected in *su1-Ref* is W578, which is present in all isoamylases examined to date from plants and bacteria except that from *E. coli* (Beatty et al., 1999). W578R, therefore, is likely to cause the defect in the SU1 isoamylase-type DBE in *su1-Ref* mutants.

Structure of the mRNA transcripts from *su1-st*

The molecular nature of the *su1-st* mutation also was characterized, again beginning with RT-PCR amplification of mRNA from homozygous mutant kernels. Primers 797 and JD02 from the Su1 cDNA sequence, which correspond to regions within exon 3 and exon 11 of the genomic sequence, respectively, were predicted to amplify a fragment of 800 bp (Beatty et al., 1997) (Fig. 2.3A). For *Su1* and *su1-Ref*, a fragment of the predicted size was

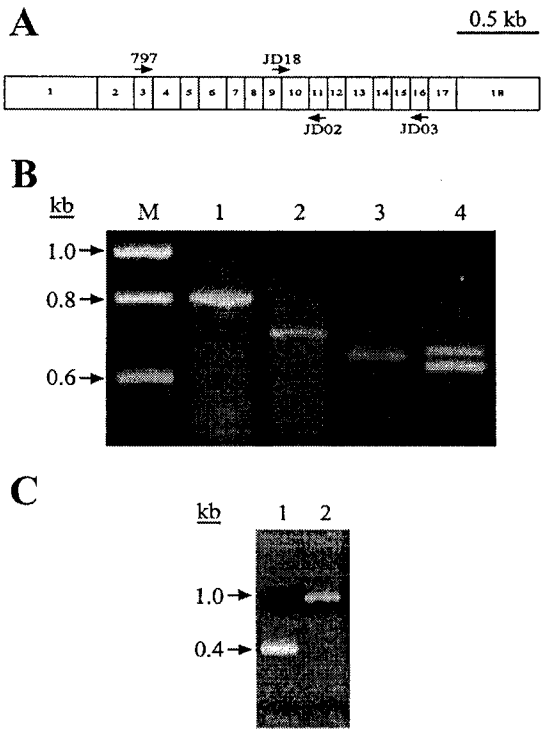

Figure 2.3. PCR and RT-PCR analyses. **A.** Relative positions of oligonucleotide primers within the Su1 cDNA. Exons comprising the Su1 cDNA are represented by numbered boxes. Primers utilized for the detection of alternatively spliced transcripts are indicated by arrows above and below the boxes (depicting sense and antisense sequences, respectively). **B.** RT-PCR amplifications. DNA was reverse-transcribed from purified mRNA isolated from 20 DAP whole kernels homozygous for *Su1* (lanes 1 and 3) and *su1-st* (lanes 2 and 4). Primer pair 797/JD02 was used for the amplifications in lanes 1 and 2, and primer pair JD03/JD18 was used for the amplifications in lanes 3 and 4. **C.** PCR amplification of genomic DNA from seedling tissue homozygous for *Su1* (lane 1) and *su1-st* (lane 2) with primer pair JD19/JD20 to amplify the region of the gene containing the *Toad* element.

amplified (Fig. 2.3B, lane 1; and data not shown). The major RT-PCR product from *su1-st* kernels, however, was approximately 100 bp smaller than that obtained from wild type kernels, although a minor product near the expected size also was seen (Fig. 2.3B, lane 2). Sequence analysis of the ~700 bp major product revealed that in this mutant mRNA, the splice donor site of exon 9 has been joined precisely to the acceptor site of exon 11, and that exon 10 is completely lacking. Loss of this 127-nucleotide exon would result in a shift in the

open reading frame within exon 11 and the introduction of a premature translational stop codon. Therefore, this transcript, designated as transcript type I, is predicted to result in the translation of a truncated, non-functional polypeptide.

The preceding observation was difficult to reconcile with the fact that *su1-st* mutants display a kernel morphology and chemical phenotype quite distinct from those of the confirmed null mutation *su1-R4582::Mu1* (Fig. 2.1). To search for minor transcripts in which exon 9 was correctly spliced to exon 10, a RT-PCR primer that bridges this splice donor site and acceptor site was synthesized. Kernel RNA was amplified by RT-PCR using this primer, JD18, and the downstream primer JD03 from within exon 16 (Fig. 2.3A). This resulted in two distinct *su1-st* mutant cDNA products, one slightly smaller than that obtained when *Su1* kernel RNA was amplified with this primer pair, and the other slightly larger (Fig. 2.3B, lanes 3 and 4). These two transcripts are designated type II and type III, respectively. The observation of multiple *su1-st* transcripts, none of which is the size of the wild type transcript, suggests that alternative splicing occurs in pre-mRNA transcripts from the *su1-st* locus.

To further investigate the apparent deletion of exon 10 in transcript I, genomic DNA from a homozygous *su1-st* plant was amplified with oligonucleotide primers JD19 and JD20, derived from intron 9 and intron 10, respectively. This primer pair is designed to amplify a sequence of 370 bp from wild type genomic DNA that includes all 127 bp of exon 10 flanked by portions of the adjacent introns. The predicted amplification product was obtained from genomic DNA from a *Su1* plant, but a product of slightly greater than 1 kb was obtained from *su1-st* genomic DNA (Fig. 2.3C). Characterization of this mutant fragment revealed the insertion of a 638 bp sequence within exon 10, as well as duplication

of 10 nt of the *Su1* sequence flanking each end of the insertion (ATTACGACAG) (Fig. 2.4). This 638 bp sequence is referred to as the "*Toad*" genetic element.

Characterization of the *su1-st* genomic sequence enabled definition of the pre-mRNA splicing events that gave rise to the type II and type III transcripts. Nucleotide sequence of the shorter cDNA amplified by the JD18/JD03 primer pair (Fig. 2.3B), corresponding to the type II transcript, revealed deletion of 18 nt within exon 10 (Fig. 2.4). This deletion extends from nucleotide 5248 in the *su1* genomic sequence through nucleotide 5265 (Genbank accession no. AF030882). The latter nucleotide corresponds to the last residue of the direct repeat adjacent to both ends of the *Toad* element. This deletion maintains the open reading frame of the mRNA, resulting in a mutant polypeptide designated $SU1^{st\text{-}II}$, that has a deletion of 6 amino acids (residues 440 - 445; GDMITT). The larger cDNA product, corresponding to the type III transcript, contains an insertion of 30 nt relative to the nonmutant sequence. This insertion corresponds exactly to the first 30 nt of the inserted *Toad* element. In this instance the reading frame is again maintained, with the insertion of 10 amino acids of the sequence EIWNHAIMLR between residues 446 and 447 of the nonmutant protein, resulting in $SU1^{st\text{-}III}$. In the type II transcript the entire *Toad* element contained in the genomic sequence is missing from the mRNA, and in the type III transcript all but 30 nt of *Toad* have been removed. Thus it appears that the insertion or deletion mutations in the type II and III transcripts are generated through aberrant pre-mRNA splicing events induced by presence of the *Toad* element.

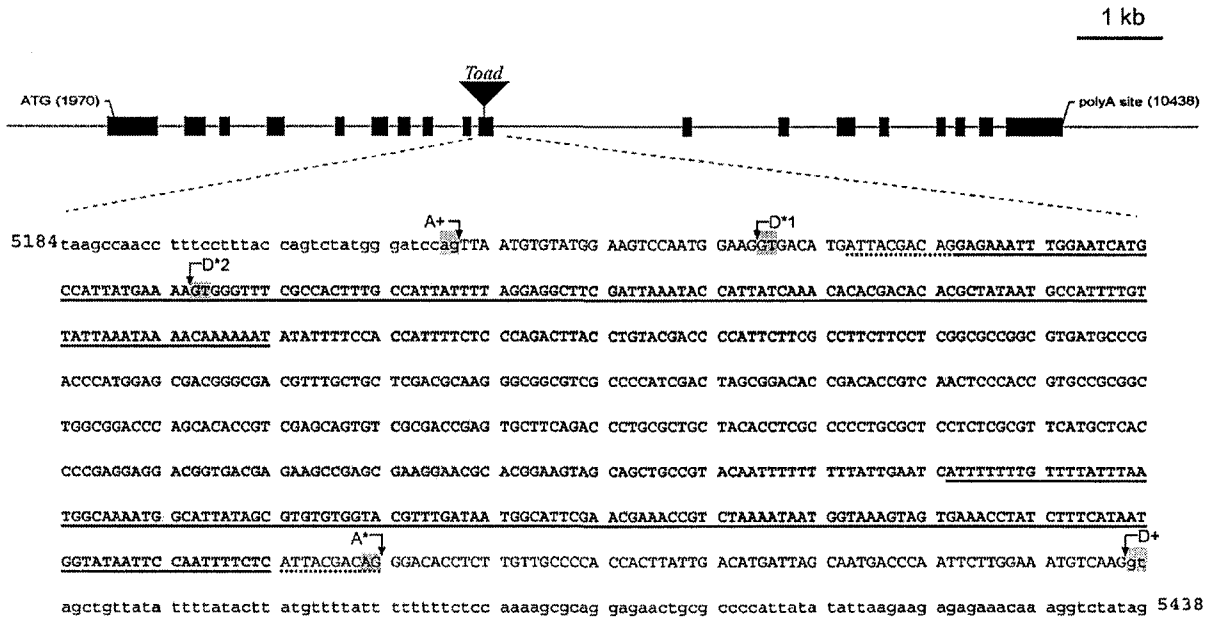

Figure 2.4 Nucleotide sequence analysis of the *su1-st* locus. The *su1-st* locus contains an insertion of the 638 bp *Toad* sequence (shown in bold) in exon 10. Intron sequence flanking exon 10 is designated by lowercase letters, and exon 10 sequence is designated by uppercase letters. Nucleotides underlined with a dotted line indicate the 10-bp target site duplication of the host sequence. Regions underlined with a solid line indicate 138bp terminal inverted repeats within the *Toad* element. The wild type acceptor site for the end of intron 9 is designated A+. The intron formed for the creation of the Type II transcript is made by joining cryptic acceptor sites D*1 to A*. This results in a deletion of 18 nt of Su1 sequence from the mature transcript and complete removal of the *Toad* sequence. The intron formed for the Type III transcript is produced by the use of D*2 as the donor site and A* as the acceptor site, removing most of the *Toad* sequence as an intron, but leaving an insertion of 30 bp in the mature mRNA. The wild type donor site for the start of intron 10 is indicated as D+. The highly conserved GT and AG dinucleotides found at the termini of most plant introns are highlighted at the indicated splice donor/acceptor sites. The nucleotides indicated correspond to the wild-type *Su1* genomic sequence (GenBank accession no. AF030882).

A novel transposon-like sequence within the *su1-st* locus

The 638 bp insertion sequence found within exon 10 at the *su1-st* locus was subjected to a BLAST search of the public databases. This search failed to identify similar sequences within any genome, indicating that the 638 bp sequence is novel. Alignment of the nucleotide sequence of the element with its reverse complement identified terminal inverted repeat (TIR) regions of 138 bp, which are 86% identical. Class II transposable elements in

flowering plants, which include all non-retrotransposons, are characterized by the presence of TIRs that range from a few to more than 200 nucleotides (Finnegan, 1989). Also, insertion of a Class II transposable element typically results in the duplication of small region of host DNA at the insertion site, such that direct host repeat sequences flank the element (Federoff, 1989). Together, these transposon-like features of *Toad* are strong indicators that the 638 bp sequence is a transposable element (the sequence of *Toad* is available as Genbank accession no. AF317693). Open reading frames of 67, 70, and 94 codons are present within *Toad*, although none of the predicted polypeptides are significantly similar to sequences in the public database.

To investigate the approximate number of copies of the *Toad* elements, or homologous elements, within the maize genome, DNA from six inbred lines was subjected to high stringency gel blot analysis with the *Toad* sequence as a hybridization probe. This analysis indicated that *Toad* is a low-copy element, with no more than two to four copies in any of the genomes tested (data not shown). In DNA from a *su1-st* plant, two *Toad* sequences were observed, one corresponding to the element at the *su1-st* locus, and the other corresponding to the single *Toad* sequence in the W64A genome. A detailed analysis of the *Toad* sequence revealed short blocks of similarity with other maize transposons, such as have been observed for many plant transposable elements (Nevers et al., 1986).

Glucan structure phenotypes

The phenotype conditioned by each *su1-* mutation with regard to the chemical properties and structures of the glucans present in developing kernels was determined. Aqueous kernel extracts were prepared from samples harvested at mid-developmental time

points from several growing seasons, and separated by centrifugation into soluble and granular phases. The quantities of sucrose, D-glucose, D-fructose, oligosaccharide, and glucan polymer in each phase were determined by specific analytical methods (Table 2.1). The carbohydrate profile conditioned by either *su1-R4582::Mu1* or *su1-Ref* is significantly altered relative to nonmutant kernels, whereas *su1-st* conditions an intermediate phenotype. The intermediate effects of the *su1-st* allele varied, however, among the samples, suggesting environmental factors may impact *su1-st* phenotypic expression.

Selected data from Table 2.1 highlight *su1* allele-specific effects on carbohydrate composition. For example, ~4% of the total carbohydrate is in the form of sucrose in both *Su1* and *su1-st* kernels harvested 30 days after pollination (DAP) from the 1997 nursery, but higher sucrose proportions are found in 20-DAP *su1-st* kernels from the 1998 and 2000 nurseries, ~14% and ~20%, respectively. Sucrose comprises 16% of the total carbohydrate in *su1-R4582::Mu1* and ~20% to 28% of the total carbohydrate in *su1-Ref* kernels. The great majority of carbohydrate in nonmutant kernels at 20- or 30 DAP, ~94%, is in the form of starch. Granular starch is reduced to various levels in *su1-st* kernels, comprising ~88% of the 30 DAP sample from 1997, ~38% of the 20-DAP sample from 1998, and 58% of the 20-DAP sample from 2000. In contrast, only ~14% to 23% of the total carbohydrate in *su1-Ref* kernels and *su1-R4582::Mu1* kernels is found in starch granules at comparable developmental stages. The relative severity of the phenotypes also is evident in the accumulation of WSP (i.e., phytoglycogen). This more highly-branched glucan accounts for ~52 to 66% of the total carbohydrate in the more phenotypically severe *su1-R4582::Mu1* or *su1-Ref* kernels, whereas in *su1-st* kernels WSP comprised ~7% of the total in the 30 DAP sample and from 26 to ~39% in the 20 DAP samples.

The structure of the amylopectin purified from granules of each mutant was analyzed by HPAEC-PAD to determine if the *su1-* mutations resulted in altered chain length distributions. Analysis of starch from 30 DAP kernels revealed that the amylopectin component in *su1-R4582::Mu1* and *su1-Ref* kernels is structurally different from wild type amylopectin (Fig. 2.5A). The amylopectin-like glucans in *su1-R4582::Mu1* and *su1-Ref* kernels are similar to each other, having more short chains, approximately 3 to 12 glucosyl units in length, and fewer intermediate-length chains, approximately 12 to 30 glucosyl units in length, relative to wild type. The *su1-st* chain length profile for amylopectin is intermediate between wild type and that of *su1-R4582::Mu1* and *su1-Ref*, with slightly more short chains and slightly fewer intermediate chains relative to wild type.

Table 2.1. Carbohydrate analysis of W64A and *su1-* mutant kernels.

			Total Carbohydrate											% Glucan Polymer	
			Glucose		Fructose		Sucrose		WSP		Starch		Total		
Sample	Year	DAP	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g	WSP	Starch
W64A	2000	20	3.7	**0.5**	3.2	0.45	43	**5.6**	5.6	**0.7**	718	**92.7**	774	0.8	99.2
W64A	1999	30	2.4	**0.3**	2	0.2	31	**3.6**	15	**1.8**	820	**94**	870	2	98
su1-Ref	2000	20	9.3	**1.5**	5.5	0.9	168	**27.8**	336	**55.6**	85	**14.1**	604	80	20
su1-Ref	1999	30	20	**3.8**	4.4	0.8	109	**20.5**	274	**52**	121	**22.8**	528	70	30
su1-4582	1998	25	15	**2**	5.5	0.8	110	**16**	465	**66.5**	103	**15**	699	80	20
su1-st	2000	20	9.1	**1.2**	7.6	1	104	**13.6**	198	**26**	444	**58**	763	31	69
su1-st	1998	20	12	**1.9**	9.3	1.5	123	**19.7**	242	**38.7**	240	**38.1**	626	50	50
su1-st	1997	30	3.6	**0.5**	2.2	0.3	30	**4**	52	**6.8**	683	**88.5**	771	7	93

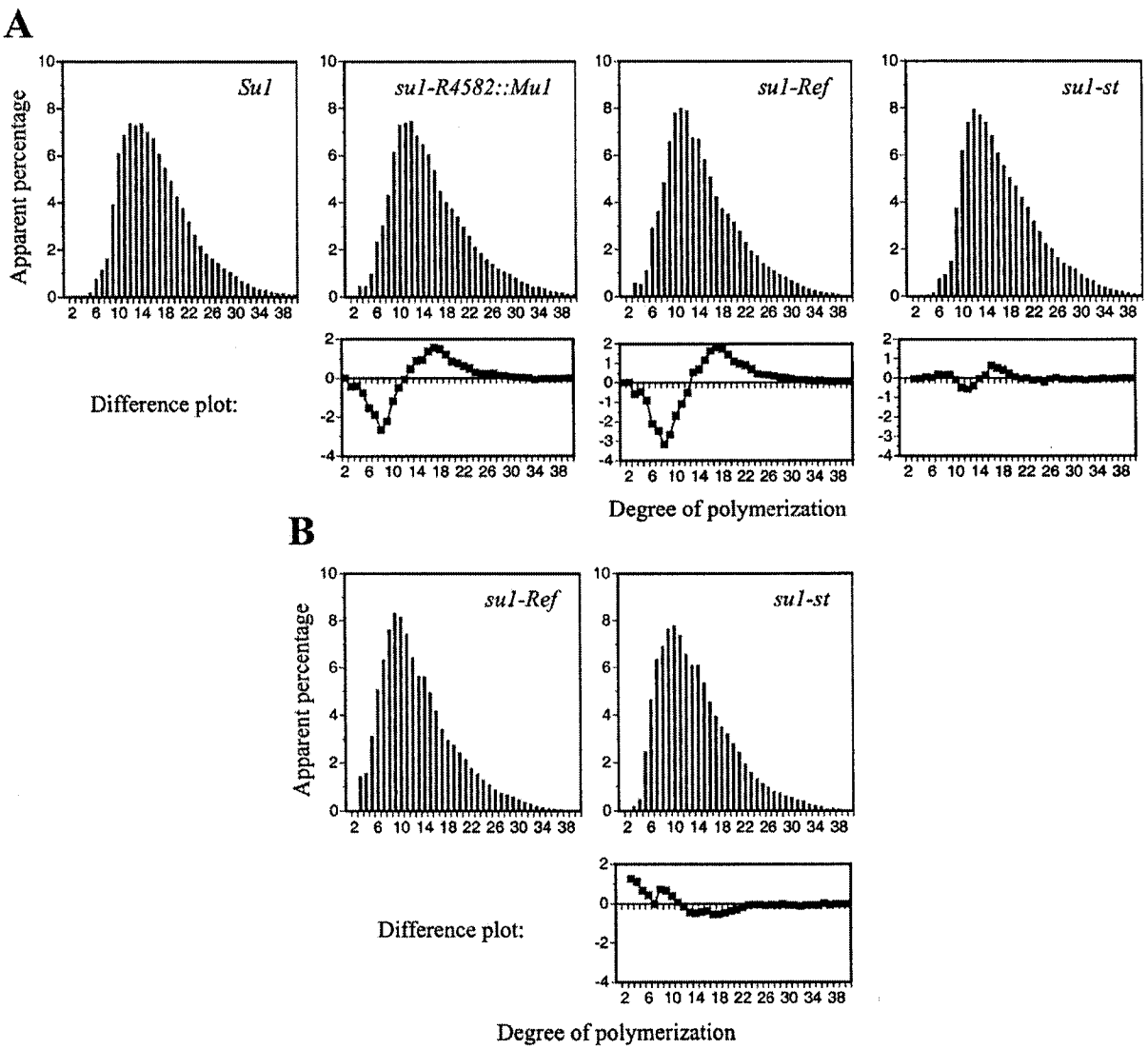

Figure 2.5. HPAEC-PAD analysis of chain-length distributions. **A.** Amylopectin from *Sul*, *sul-R4582::Mul*, *sul-Ref*, and *sul-st* homozygous kernels harvested 30 DAP was debranched with *Pseudomonas* isoamylase. Chain length distributions were determined using HPAEC-PAD and normalized to the total peak area. Differences in chain lengths of *sul-R4582::Mul*, *sul-Ref*, and *sul-st* amylopectin-like molecules relative to *Sul* amylopectin are shown in difference plots below the respective mutant profiles. **B.** WSP from *sul-Ref* and *sul-st* kernels harvested 30 DAP was debranched and separated, as described for **A**. A difference plot comparing the WSP chain distributions is below the *sul-st* profile.

Thus, the *su1-st* amylopectin-like glucan is structurally distinct from any of the others. A similar analysis of the WSP from *su1-Ref* and *su1-st* kernels also shows that these two soluble glucans have structural differences, as evidenced by a reduction in very short chains, less than six glucosyl units in length, in the *su1-st* WSP (Fig. 2.5B).

Enzyme profile phenotypes

To examine the pleiotropic effects of *su1* mutations on starch metabolizing enzyme activities, proteins from developing wild type kernels and kernels homozygous for *su1-Ref*, *su1-R4582::Mu1*, and *su1-st* were separated by native PAGE and transferred to acrylamide gels containing solubilized starch. After incubation of the activity gels (i.e., zymograms) and staining with I_2/KI, enzymes that modify starch structure are detected by alterations in the dark blue backgound color. Thus, DBE activities are visualized as light blue staining bands, BE activities as red or orange staining bands, and amylolytic activities as regions where staining is reduced to white or very light color. Initially, *su1-R4582::Mu1* and *Su1* extracts were compared on 5 cm gels (Fig. 2.6A), which showed that a series of light blue bands was completely missing in the mutant extract. These bands did not appear when red-pullulan, a pullulanase-type DBE-specific substrate, was included in the activity gels (data not shown). Considering that *su1-R4582::Mu1* is a null mutation of a gene coding for an isoamylase-type DBE (see below), these data strongly suggest that the light blue bands represent the isoamylase activity coded for by *su1*.

All three mutants also were analyzed on 15 cm activity gels, which reveal a larger number of starch metabolizing enzymes (Fig. 2.6B). In nonmutant extract, the isoamylase activity was observed as a series of diffuse, light-blue bands at the top of the gel. This broad

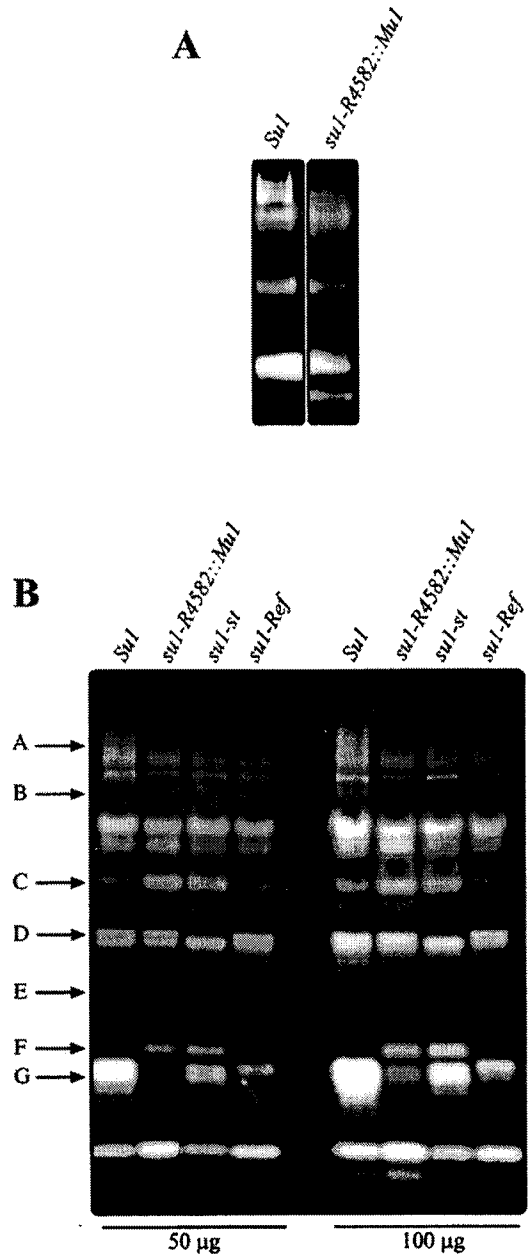

Figure 2.6. Native PAGE/activity gels (zymograms) of starch modifying activities. **A.** 25 µg total protein from *Su1* and *su1-R4582::Mu1* homozygous kernels harvested at 20 DAP were separated on a 5 cm native polyacrylamide gel, then electroblotted to a starch-containing gel. Starch modifying enzyme activities were visualized by staining with I_2/KI. **B.** 50 µg or 100 µg total protein from *Su1*, *su1-R4582::Mu1*, *su1-st*, and *su1-Ref* homozygous kernels harvested at 20 DAP were analyzed as described for A on 15-cm native PAGE/activity gels. Arrows A through G indicate starch modifying activities that exhibit allele-specific variation.

staining area labeled as arrow A is not detected in any of the three *su1*- mutants, and the light blue band labeled as arrow B also is missing in the mutants. Notably, the pattern in this region of the gel is the same in all three *su1*- mutants. Another light blue band was evident in the 15 cm gels, noted by arrow E. Duplicate gels including red-pullulan as the substrate showed an activity band of the same electrophorectic mobility, demonstrating that this signal indicates the pullulanase-type DBE (data not shown). This pullulanase-type enzyme activity was reduced in all three mutants relative to wild type, consistent with previous characterizations of DBEs in *su1-Ref* (Pan and Nelson, 1984; Beatty et al., 1999) and *su1*- mutants of rice (Kubo et al., 1999).

The *su1*- mutations also result in a wide variety of allele-specific changes in other starch modifying activities, as exhibited by altered staining intensity or protein mobility. An apparent BE activity (Fig. 2.6B, arrow C), as indicated by the red color of the band, is increased in staining intensity in *su1-R4582::Mu1* and *su1-st* extracts compared to wild type, although this activity is not affected by *su1-Ref.* Also, a new apparent amylolytic activity, as suggested by the white color of the band, appears specifically in the *su1-R4582::Mu1* and *su1-st* mutants (arrow F). An modifying activity (arrow D) corresponding to SBEIIa is not detected specifically in *su1-st* extracts. Furthermore, a doublet of apparent amylolytic activity (arrow G) is reduced in intensity in all three *su1*- mutant extracts, and the lower of these two bands is differentially affected by *su1-Ref.*

Discussion

In this study, the direct molecular effects of three different *su1*- mutations on the isoamylase-type DBE specified by the gene were clearly determined. For *su1-R4582::Mu1*,

the structure of the gene and lack of transcript indicate that the SU1 protein is completely missing in homozygous mutants. In contrast, both *su1-Ref* and *su1-st* produce transcripts that code for mutant SU1 polypeptides. Expression of *su1-Ref* appears to be normal at the level of mRNA accumulation, although the polypeptide abundance is reduced compared to wild type (Beatty et al., 1999). Thus, the $SU1^{Ref}$ mutant protein may be unstable relative to the wild type SU1 protein. The fact that the kernel appearance and the chemical structure phenotype conditioned by *su1-Ref* closely resemble that caused by the null allele suggests that this mutant protein lacks enzymatic activity. SU1 isoamylase-type DBE activity was, in fact, shown to be severely reduced both in partially purified extracts (Beatty et al., 1999) and in the activity gels in this study. This loss of enzymatic activity is likely to result from substitution of the hydrophobic residue tryptophan in SU1 region IS-XII, which is nearly universally conserved in all known isoamylase-type enzymes, by the charged residue arginine.

In spite of their phenotypic similarities, differences do exist in the effects of *su1-Ref* and *su1-R4582::Mu1*. In particular, the former is dominant to *su1-st* with respect to kernel phenotype, whereas the latter is recessive. These differences may be explained by a complex effect of the *su1-st* allele on SU1 protein expression. The steady-state *su1-st* mRNA level is significantly reduced compared to wild type, and at least three different transcripts are produced from the mutant gene. Only two of these transcripts, type II and type III, are capable of producing full SU1 polypeptides, $SU1^{st\text{-}II}$ and $SU1^{st\text{-}III}$. At least one of these proteins must be present in order to explain the dominance of *su1-st* over the null allele *su1-R4582::Mu1*, although whether both forms accumulate cannot be discerned from these data. Furthermore, the mild kernel phenotype and intermediate chemical structure

phenotype of homozygous *su1-st* kernels suggests that at least one of the $SU1^{st\text{-}II}$ and $SU1^{st\text{-}III}$ polypeptides possesses some enzymatic activity. This is likely to be a low level of activity, however, because the zymogram analysis showed the same result for *su1-st* and *su1-R4582::Mu1*. Alternatively, *su1-st* might not produce any active isoamylase-type DBE, in which case the partial function of this allele would have to be explained by a structural property of the polypeptide independent of starch hydrolytic activity.

The dominant/recessive relationships of the three mutant *su1* alleles are likely to be explained by the multimeric nature of the SU1 holoenzyme. The rice isoamylase-type DBE exists as a high molecular weight homomultimer, estimated to comprise four to six monomers (Fujita et al., 1999), and the maize enzyme also migrates as a high molecular weight complex in gel filtration chromatography (Beatty et al., 1999). Because *su1-R4582::Mu1* is a null allele, *su1-st/su1-R4582::Mu1* heterozygous kernels are expected to assemble a relatively small amount of SU1 isoamylase in a complex comprising $SU1^{st\text{-}II}$ and/or $SU1^{st\text{-}III}$, as would occur in homozygous *su1-st* kernels. Although such a complex would be reduced in abundance, it is likely to be enzymatically active and to provide some level of the DBE function needed for starch biosynthesis. The dominance of *su1-Ref* over *su1-st* most likely is explained by multimeric complexes containing $SU1^{Ref}$, $SU1^{st\text{-}II}$ and/or $SU1^{st\text{-}III}$, which we propose are inactive despite the presence of some potentially functional subunits. According to this hypothesis, the abundance of the $SU1^{st}$ subunits must be low relative to $SU1^{Ref}$, such that the occurrence of homomultimers lacking any $SU1^{Ref}$ subunits is very rare.

The potential production of two distinct mutant polypeptides from the *su1-st* gene is the consequence of the *Toad* element insertion within exon 10. The type I transcript results

from the skipping of exon 10 and the inserted *Toad* element during splicing. The splice donor and acceptor sites that border exon 10 are intact in the mutant, however, and the context of the sequence is such that they are not recognized by the splicing machinery. Plant introns normally are AU-rich, although the exons surrounding them are GC-rich (Wiebauer et al., 1988). Intron recognition is thought to occur in part because of the high A+U composition of a sequence, but also because of discrete borders that separate AU-rich from GC-rich regions (Luehrsen et al., 1994; Lorkovic et al., 2000). The Toad TIRs are AU-rich (69%), so that their insertion into the small exon 10 sequence may mask the AU-rich/GC-rich borders that exist in the nonmutant su1 gene. This would creats a new, larger intron containing the exon 10 sequences. Additional examples of exon skipping and/or intron creation owing to AU-rich transposons are known. These include insertion of *Ds2* within the *shrunken2* (*sh2*) locus, creating *sh2-m1* (Giroux et al., 1994), insertion of *dSpm* within the *A2* locus, creating *a2-m1* (Menssen et al., 1990), and insertion of *dSpm* at the *bronze1* locus, creating *bz1-m13* (Kim et al., 1987).

The AU-rich composition of the *Toad* TIRs also is likely to activate cryptic splice sites within the *Su1* sequence or the *Toad* element that result in formation of the type II and type III transcripts. Determination of 5' and 3' splice sites is mediated by downstream and upstream AU-rich tracts, respectively (Lou et al., 1993; McCullough et al., 1993). The *Su1* sequence normally contains both the D*1 cryptic donor site and the A* cryptic acceptor site (see Fig. 4), although neither is recognized by the splice machinery. Repositioning of the A* sequence downstream of the *Toad* insertion, as part of the host site duplication, changes the AU-environment of the sequence and enlarges the physical distance between it and either

cryptic donor site (D*1 or D*2). Thus, these sites are utilized by the splicing machinery only when *Toad* is present.

The zymogram profiles of enzyme activities clearly show that the *su1* mutant alleles differ in their effects on specific BE activities and the activity of one or more amylolytic enzymes (Fig. 2.6). These observations suggest that the $SU1^{Ref}$ mutant polypeptide does have some functionality with respect to other enzymes, even if it lacks enzymatic function. One possible explanation is that either the SU1 or $SU1^{Ref}$ polypeptide can inhibit or alter the activities marked by arrows C and F in Fig. 6, whether or not the proteins constitute an active isoamylase-type DBE. In contrast, *su1-R4582::Mu1* causes complete absence of the SU1 protein, which may explain the changes in these activities. Similarly, the *su1-st* mutation most likely results in accumulation of only a small amount of $SU1^{st\text{-}II}$ and/or $SU1^{st\text{-}III}$ polypeptide, and thus is likely to condition the same pleiotropic effects as the null allele. The unique effect of *su1-st* on the loss of a starch modifying activity indicated by arrow D in Figure 6, which is distinct from that of *Su1*, *su1-Ref*, or *su1-R4582::Mu1*, is likely to arise from a structural property of $SU1^{st\text{-}II}$ and/or $SU1^{st\text{-}III}$. This argument derives from the fact that this band does not vary depending on SU1 activity, because it is the same in the null mutant and the wild type. In summary, we propose that the overall function of the *su1* gene depends on 1) the level of enzymatic activity, 2) the level of the polypeptide independent of enzyme activity, and 3) specific structural properties of the SU1 polypeptide, again unrelated to enzyme activity.

The *su1-Ref* and *su1-R4582::Mu1* mutations clearly affected the structure of amylopectin in starch granules with regard to the chain length distribution (Fig. 2.5). These data are consistent with the analysis of rice *su1-* mutants (Kubo et al., 1999), but differ from

the analysis of Arabidopsis leaf starch in which an isoamylase mutation caused phytoglycogen accumulation without altering the structure of residual amylopectin (Zeeman et al., 1998). Both direct and indirect roles of DBE function in amylopectin biosynthesis have been proposed to account for the simultaneous occurrence of amylopectin and WSP in isoamylase-deficient mutants (Zeeman et al., 1998; Fujita et al., 1999; Myers et al., 2000). As far as can be determined from the activity gel profile, BEs are not altered significantly by *su1-Ref*, suggesting that changes in these enzymes are not responsible for the altered amylopectin structure. The change in chain length distribution is consistent with the hypothesis that the isoamylase-type DBE, and/or the pullulanase-type enzyme, directly modifies a precursor to amylopectin prior to its crystallization into the granule. One possibility is that the DBE(s) preferentially remove chains of a certain length, or chains positioned in some specific way within the newly branched region of the growing amylopectin molecule. An indirect role of the DBE, however, is not ruled out by these data. For example, the presence of phytoglycogen might alter the function of an SS or a BE, or altered supply of the substrates ADP-Glucose or maltooligosaccharide might affect an SS activity such that it synthesizes chains of different lengths than normal.

The transcript characterization, the activity gel data, and the recessiveness of *su1-st* relative to *su1-Ref*, indicate that the level of isoamylase-type DBE activity is very low in *su1-st* mutants. The relatively mild kernel- and chemical structure phenotypes of *su1-st* homozygotes and *su1-st/su1-R4582::Mu1* heterozygotes, therefore, support the notion that only a very small amount of SU1 isoamylase is sufficient for synthesis of significant quantities of normal or near normal amylopectin. In nonmutant endosperm there may be an excess of isoamylase-type DBE activity over what is needed to produce wild type levels of

amylopectin. A major reduction in this enzyme in *su1-st* mutants may still leave enough function to produce significant quantities of starch, as was seen in the 30 DAP kernels from the 1997-growing season. The variability in *su1-st* expressivity may be explained by environmental factors affecting the threshold level of isoamylase-type DBE activity required for normal starch biosynthesis.

This study has provided new information about the effects of mutations in the isoamylase-type DBE. The observation of numerous allele-specific effects on starch structure and the activities of other starch modifying enzymes implicates both enzymatic and non-enzymatic features of the SU1 polypeptide in starch biosynthesis. We expect that the pleiotropic effects of the *su1-* mutations are indicative of fundamental aspects of the starch biosynthetic mechanism.

Materials and Methods

Plant Materials and Nomenclature

The nomenclature follows the standard maize genetics format (Beavis et al., 1995). The mutant allele *su1-st* arose spontaneously in a population of Krug's yellow dent corn (Dahlstrom and Lonnquist, 1964) and was generously provided by Dr. Oliver Nelson (University of Wisconsin, Madison). All *su1-* mutant alleles were introgressed into the W64A inbred background by five generations of back crossing to the standard line.

RNA Gel Blot Analyses

Total RNA was isolated from maize kernels harvested 20 DAP and subjected to gel blot analysis as described previously (James et al., 1995; Gao et al., 1996). RNA gel blots

were hybridized with a 387 bp antisense RNA probe from the 3' end of the Su1 cDNA. The probe was synthesized as an in vitro run-off transcription product using the StripEZ T7 kit (Ambion catalog no. 1362). The transcription template was plasmid pSu1(BS), linearized at the unique *Bgl*II site within the Su1 cDNA. The RNA probe corresponds to nt 2608 to nt 2221 of the Su1 cDNA sequence (GenBank accession no. U18908) running in the antisense direction.

PCR, RT-PCR, and DNA Sequence Analysis

Sequencing of the *su1-Ref* coding sequence followed PCR and RT-PCR amplifications. PCR amplification of *su1-Ref* genomic DNA was used to obtain 5' end sequence information with the primer pair JD01 (5'-GGC TCC CTC CCC TCC ACT TCC-3') and 452 (5'- GGG ATC ATA CCA GCC ATT TGA-3'). Downstream sequence information for *su1-Ref* and *su1-st* was obtained following RT-PCR amplification of mRNA using the following primer pairs: 797 (5'-CTC AAA TGG CTG GTA TGA-3') and JD02 (5'-CCA TTC CAC TCT GAC CAA ACG-3'); MB02A (5'-GGG GAA AAT CAT AAT CTT A-3') and MB02N (5'-TGG TAG ACG GTG ACA GCA-3'); JD03 (5'-ATT GTT CTT CCT TCT TAT CCC-3') and MB03D (5'-TTG GTC AGA GTG GAA TGG-3'); JD18 (5'-ACC AGA GGA TGC AGT CTA TG-3') and JD03. Primers JD19 (5'-TCC TTA TTT TGT CTT TTT AGG CAT TCT-3') and JD20 (5'-GCA GAT GTT GGG AGG GTT AGT GTC T-3') were used for PCR amplification of *su1-st* genomic DNA containing the *Toad* sequence. Template mRNA for RT-PCR was purified from total RNA using the PolyA+ Tract mRNA isolation system (Promega catalog no. Z5300). The HF One-step RT-PCR System (Stratagene catalog no. 600164) was used for the RT-PCR. Sequences were determined

directly from the PCR products, except for the type II and type III transcripts produced from *su1-st* by the JD18/JD03 primer pair. In those instances the PCR products were first cloned using the pGEM-Teasy vector system (Promega catalog no. A1360) and then sequenced from the recombinant plasmids. The amplification product from the JD19/JD20 primer pair, containing the *Toad* element and flanking *Su1* genomic sequences, also was cloned in the vector system, producing plasmid pJD19. Computational analyses were performed using the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI).

DNA Gel Blot Analysis

Genomic DNAs were isolated from seedling leaves of a homozygous *su1-st* plant in the W64A background and inbred lines W64A, Oh43, Mo17, B73, and F_1 hybrids B77/B79 and Q66/Q67 according to published procedure (Dellaporta et al., 1983), digested with *Bam*HI and *Eco*RI, electrophoresed, and transferred to nylon membranes. The blots were hybridized at 65°C in a buffer of 5X SSC, 5X Denhardt's solution, 0.1% SDS, and 10 $\mu g\,mL^{-1}$ sonicated, denatured salmon sperm DNA. The hybridization probe was the 0.5 kb *Eco*RI/*Pvu*II fragment from pJD19 (containing the central portion of the *Toad* element and one TIR), labeled with ^{32}P using the RTS Radprime Kit (Gibco-BRL catalog no. 10387-017). Blots were washed one time in 2X SSC, 0.1% SDS at 65°C for 15 min and once in 0.2X SSC, 0.1% SDS at 65°C for 15 min.

Carbohydrate Extraction and Quantification

Immature kernels were harvested 20 - 30 DAP and stored at -80°C. Insoluble starch and soluble carbohydrates were extracted from five whole kernels as follows. After removal of the pericarp and embryo by dissection, endosperm tissue was ground in H_2O in a chilled

mortar and pestle, and the volume was brought to 4 ml. Water was removed from a 1 mL portion of the homogenate by drying in an oven at 70°C, and the kernel dry weight was determined from the remaining material. The remaining 3 mL portion of the homogenate was centrifuged at 10,000*g* for 5 min. The pellet was washed in cold H_2O and again centrifuged at 10,000*g* for 5 min. The supernatant fractions from both centrifugations, containing soluble carbohydrates, were pooled and incubated in a boiling water bath for 10 min. The pellet fraction, consisting mostly of insoluble starch granules, was dissolved in 100% DMSO and incubated in a boiling water bath for 30 min.

Total glucan oligomer or polymer content was measured in terms of mg carbohydrate/g dry weight following complete digestion of the insoluble starch fraction and the soluble carbohydrate fraction with amyloglucosidase. These assays employed a commercial glucose assay kit (Boehringer-Mannheim catalog no. 207748) according to the manufacturer's protocol. The quantities of sucrose, D-fructose and D-glucose were measured in terms of mg carbohydrate/g dry weight by specific enzymatic assays (Boehringer-Mannheim catalog no. 716260).

Starch Structural Analysis

Approximately 20 mg glucose equivalents of solubilized granular starch in 100% DMSO was precipitated by overnight incubation in 4 volumes absolute ethanol at -20°C. During this step, we presume that lipids have been removed from the carbohydrate fraction. After centrifugation at 2,000*g* for 5 min, the pellet was dissolved in 2 mL of 10 mM NaOH. Amylopectin and amylose were separated by gel permeation chromatography on Sepharose CL-2B columns (1.8 m height; 1.8 cm diameter). Samples were eluted with 10 mM NaOH

at a rate of 30 mL/h. Aliquots of each 3 mL fraction (100 μL) were mixed with 400 μL of I_2/KI (0.67% (w/v) I_2 and 3.33% (w/v) KI) solution, and maximal absorbance was determined by measuring the spectrum from 400 nm to 700 nm. Fractions containing amylopectin or amylose were pooled, dialyzed against H_2O at 4°C, lyophilized, and stored at room temperature.

Characterization of the chain length distribution by HPAEC-PAD chromatography was performed as previously described (Fontaine et al., 1993). Briefly, the lyophilized amylopectin obtained from the gel permeation column was dissolved in 90% DMSO, quantified, diluted to 1 mg mL^{-1}, and digested to completion with 0.2 U mL^{-1} *Pseudomonas* sp. isoamylase (Megazyme catalog no. E-ISAMY) during an overnight incubation at 45°C. Samples (50 μL) were injected into the Dionex HPAEC-PAD system for characterization of the chain length distribution. The area under each peak of chain length 3 - 40 glucose units was integrated and the apparent frequency of each chain length was calculated as the percentage of total peak area.

Activity Gel Analysis of Starch Modifying Enzymes

Frozen kernels (5 g) were ground to a fine powder in liquid nitrogen with a mortar and pestle, and the tissue was suspended in 5 mL of buffer containing 50 mM sodium acetate, pH 6, 20 mM DTT. All of the lysates were centrifuged at 50,000*g* for one hour at 4°C, which removes phytoglycogen from the *su1*- mutant extracts. The supernatant was stored at -80°C. Total soluble proteins (50 μg or 100 μg) were separated on a native polyacrylamide gel (16 cm × 20 cm × 0.15 cm). The resolving gel contained 7% (w/v) acrylamide (29:1 acrylamide-bisacrylamide was used throughout) and 375 mM Tris-HCl,

pH 8.8. The stacking gel contained 4% (w/v) acrylamide and 63 mM Tris-HCl, pH 6.8. Electrophoresis was conducted at 4°C, 25 V cm^{-1} for 4 h using a Protean II cell (Bio-Rad) in an electrode buffer of 25 mM Tris, 192 mM glycine, pH 8.8, and 2 mM DTT. At the end of the run, the gel was electroblotted to a polacrylamide gel of the same size containing 7% acrylamide, 0.3% (w/v) potato starch (Sigma), and 375 mM Tris-HCl, pH 8.8. The transfer was performed overnight at 20 V in the electrode buffer at room temperature. Starch metabolic activities were observed by staining the gel with I_2/KI solution, and the gel was photographed immediately. The 5 cm activity gels were identical except that the Mini-Protean II cell (Bio-Rad) was used, 25 μg of the sample was applied, and electrophoresis was for 45 min.

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CHAPTER 3. MUTATIONAL ANALYSIS OF THE PULLULANASE-TYPE STARCH DEBRANCHING ENZYME OF MAIZE INDICATES MULTIPLE FUNCTIONS IN STARCH METABOLISM

A paper published in *The Plant Cell*[1]

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Abstract

Plants contain two types of α(1→6) glucan hydrolase (starch debranching enzyme; DBE). Mutations affecting the pullulanase-type DBE have not been described, although defects in isoamylase-type DBE, known in many plant species, indicate a function in starch biosynthesis. This report describes a null mutation of a pullulanase-type DBE gene, a *Mutator* insertion in maize *Zpu1*. Plants homozygous for the *zpu1-204* mutation are impaired in transient and storage starch degradation. Thus, hydrolytic activity of pullulanase-type DBE contributes to starch catabolism. Developing *zpu1-204* endosperm accumulates branched maltooligosaccharides not found in wild-type, and is deficient in linear maltooligosaccharides, indicating that the pullulanase-type DBE functions in glucan hydrolysis during kernel starch formation. Furthermore, in a background deficient in isoamylase-type DBE, *zpu1-204* conditions a significant accumulation of phytoglycogen in the kernel, which is not seen in wild-type. Pullulanase-type DBE, therefore, partially

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compensates for the defect in isoamylase-type DBE, suggesting a function during starch synthesis as well as degradation.

Introduction

The central role served by starch in the metabolic strategy of plants requires that its production and degradation be strictly coordinated events. Starch accumulates in photosynthetic tissues when light is available, but in the dark the metabolic flux changes so that starch is degraded and serves as a source of reduced carbon. Distinct controls operate in reproductive and perenniating tissues (e.g. seed and tuber), which synthesize starch continuously throughout the diurnal cycle. Degradation commences in such storage tissues during germination and sprouting to supply metabolites and energy to the developing plant before it attains photoautotrophy. Starch is involved in many other physiological processes. In the gravitropic response of roots and shoots, for example, sedimentation of amyloplasts is believed to be the sensing mechanism for the orientation of the plant with respect to gravity (Chen et al., 1999). The metabolism of starch, therefore, is essential to the development and fitness of plants. Most of the molecular detail regarding the biochemical mechanisms that accomplish and control starch anabolism and catabolism remains to be discerned.

All plants characterized to date contain two remarkably conserved types of $\alpha(1\rightarrow6)$ glucan hydrolase (i.e., starch debranching enzyme, or DBE) with as yet undefined function(s) in starch metabolism. The two classes, pullulanase-type DBE (also known as R-enzyme or limit-dextrinase) and isoamylase-type DBE, are defined by sequence similarity and *in vitro* substrate specificity. These enzymes appear to have been separately conserved through the evolution of prokaryotes and higher plants, because each is more closely related

to a bacterial protein than to the other type in the same plant species. Each DBE type, therefore, is likely to provide a unique function in the processes of carbohydrate storage and utilization. In maize the pullulanase-type DBE, termed ZPU1, is coded for by the *Zpu1* gene (Beatty et al., 1999). At least three isoamylase-type DBE genes are present in the maize genome. *Sugary1* (*Su1*) codes for the major activity present in maize endosperm (James et al., 1995; Rahman et al., 1998), and two additional isoamylase-type DBE coding sequences are defined by the Iso2 and Iso3 cDNAs (J. Dinges, unpublished results) (Genbank accession nos. AY172633 and AY172634, respectively).

The DBEs are part of a large network of proteins that participate in starch metabolism, which includes ADP-Glc pyrophosphorylase, starch synthase (SS), starch branching enzyme (SBE), disproportionating enzyme, α-amylase, β-amylase, starch phosphorylase, and α-glucosidase. The complexity of starch metabolism is evident from the fact that multiple isoforms exist in plants for all of these classes of enzymes. The Arabidopsis genome, for example, codes for more than 30 such proteins. Understanding of the functional contribution of each enzyme isoform to starch assembly and disassembly will help to explain why so many proteins are needed for this process in plants, compared to the few required for glycogen metabolism in other kingdoms.

A straightforward hypothesis based on the hydrolytic activity of DBEs is that they function in the degradation of starch. If so, then mutations affecting such enzymes might be identified through starch excess phenotype, e.g., accumulation of abnormally high levels in plants that fail to fully degrade leaf starch in the dark. Examples from Arabidopsis are *sex4*, *dpe1*, and *sex1*, which affect, respectively, an amylase (Zeeman et al., 1998a), a D-enzyme (Critchley et al., 2001), and the R1-homolog, a dikinase speculated to control the rate of

starch degradation (Yu et al., 2001; Ritte et al., 2002). Mutations in other starch degradative enzymes have no noticeable effect on starch synthesis or degradation, for example Arabidopsis *ram1*, which codes for the major form of β-amylase (Laby et al., 2001). To date, a mutation affecting pullulanase-type DBE has not been identified in a starch excess screen or any other, so that clues to its function are not available from genetic analysis. The lack of a known pullulanase-type DBE mutation cannot be explained by gene duplication, because in maize the gene *Zpu1* is present in only one copy (Beatty et al., 1999), and the complete rice and Arabidopsis genome sequences also contain only one such gene (Tabata et al., 2000; Yu et al., 2002).

In contrast to pullulanase-type DBE, mutations affecting an isoamylase-type DBE are observed frequently in many plant species including maize (James et al., 1995), rice (Nakamura et al., 1996b), barley (Burton et al., 2002), *Chlamydomonas* (Mouille et al., 1996), and Arabidopsis (Zeeman et al., 1998b). The function of this isoamylase-type DBE is not evident from analysis of the mutant phenotypes. Contrary to the expectation of excess starch owing to loss of a catabolic function, there is a deficiency in starch production and a concomitant accumulation of phytoglycogen, which does not occur in wild-type plants. Phytoglycogen resembles glycogen in structure, and is similar in many respects to amylopectin, but lacks the structural organization that allows crystallization into insoluble granules. One hypothesis to explain this phenotype is that this DBE is directly involved in determination of the architectural structure of amylopectin (Ball et al., 1996; Myers et al., 2000). Another hypothesis proposes that isoamylase-type DBE degrades soluble glucans that would otherwise interfere with amylopectin synthesis by drawing off enzymes and substrate from the productive pathway (Zeeman et al., 1998b; Smith, 2001). A third model

proposes that DBE activity is required for proper starch granule initiation (Burton et al., 2002). Thus, the function of isoamylase-type DBE in starch biosynthesis remains unclear. Furthermore, the fact that mutation of an isoamylase-type DBE affects starch formation does not rule out the possibility that the enzyme also functions in starch degradation.

Even less is known about the physiological function of the pullulanase-type DBE. Owing to lack of a defined mutation, it was not known whether this enzyme also participates in starch assembly, or is involved only in catabolism, or has a dual function. To address this question, this study applied reverse genetics to identify maize plants containing a null mutation of the *Zpu1* gene. The mutant plants completely lack pullulanase-type DBE activity, a condition that has not been reported previously. ZPU1 is required for normal rates of starch degradation in leaves and seeds, and thus clearly plays a catabolic role. Function of ZPU1 during starch anabolism also is indicated, based on observation that the mutation conditions accumulation of phytoglycogen in endosperm tissue in a specific genetic background. Thus, the pullulanase-type DBE appears to perform a function both in the biosynthesis of starch and the degradation of granules.

Results

Isolation and Characterization of *zpu1-* Mutants

The Trait Utility System for Corn (TUSC, Pioneer Hi-Bred International, Inc.) (Bensen et al., 1995) was used to identify *Mutator* (*Mu*) transposable element insertions in the *Zpu1* gene (Beatty et al., 1999). Pool screening was conducted using *Zpu1*-specific primers, pulA and pulB (Figure 3.1A), and the degenerate *Mu* terminal inverted repeat primer, 9242. Specific PCR amplification products requiring one *Zpu1* primer and the *Mu* primer were

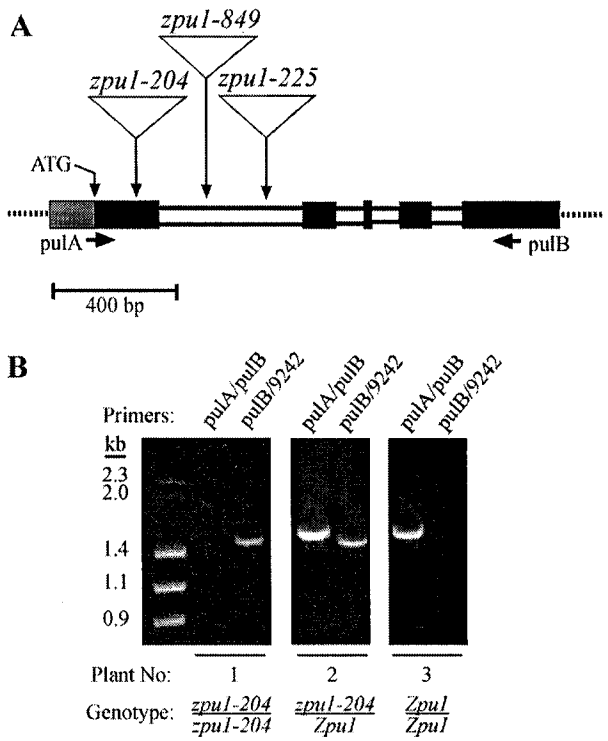

Figure 3.1. Molecular structure of *zpu1-* mutant alleles. **A.** The relative positions of *Mu* transposable elements within the *Zpu1* gene. The allele *zpu1-204* contains a *Mu* element in the first exon of the gene, downstream of the ATG start codon. The alleles *zpu1-225* and *zpu1-849* contain insertions in the first intron of the gene. The positions of the PCR primers pulA and pulB are indicated by arrows. Exons are indicated by black boxes. **B.** Genotyping of plants was determined using PCR. The pulA/pulB primer pair amplifies the wild-type *Zpu1* allele from genomic DNA (~1.6 kb), while the pulB/9242 primer pair amplifies the mutant *zpu1-204* allele (~1.4 kb). Three representative plants are shown. Plant 1 is homozygous for *zpu1-204*, plant 2 is heterozygous *zpu1-204/Zpu1*, and plant 3 is homozygous *Zpu1*.

identified in nine TUSC F_1 leaf DNA pools. PCR screening in a successive generation (F_2) revealed that three of these *Mu* insertions in *Zpu1* are transmissible through the germ line. These mutations were designated *zpu1-204*, *zpu1-225*, and *zpu1-849*, such that the allele number indicates the TUSC pool from which each was originally identified.

The location of the *Mu* transposable element within the *zpu1* locus was determined by sequencing of the pulB/9242 and pulA/9242 PCR products. First, the pulA/pulB PCR

amplification product from wild-type (W64A) genomic DNA (Figure 3.1B) was sequenced. Comparison of this genomic sequence to the Zpu1 cDNA sequence revealed four introns within the 5' region of the *Zpu1* gene (Figure 3.1A). The sequences of the pulB/9242 and pulA/9242 fragments immediately flanking the *Mu* termini were then compared to the wild-type genomic sequence, thus identifying the transposon insertion sites. The alleles *zpu1-225* and *zpu1-849* arise from *Mu* element insertions in the first intron. The *zpu1-204* mutation, however, contains a *Mu* element inserted in the first exon at nt 189 in the Zpu1 cDNA (Genbank Accession No. AF080567). This mutation was analyzed further owing to the likelihood that disruption of the coding sequence would inactivate *Zpu1* function.

The *zpu1-204* mutation exhibited simple Mendelian inheritance. Amplification of a 1.6 kb fragment with the pulA/pulB primer pair reveals the presence of the wild-type allele *Zpu1*, and production of a 1.45 kb fragment using the pulB/9242 primer pair indicates that the mutant allele *zpu1-204* is present. Figure 3.1B shows the genotypes of three representative plants grown from randomly selected kernels produced by self-pollination of a heterozygous *zpu1-204/Zpu1* plant. Hundreds of progeny plants from such crosses have been examined, and the expected ratio of 25% *Zpu1* homozygotes, 25% *zpu1-204* homozygotes, and 50% *Zpu1/zpu1-204* heterozygotes was closely approximated in the actual PCR genotype data.

zpu1-204 is a Null Allele

Gene expression from the *zpu1* locus was characterized at the level of mRNA accumulation using reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from developing wild-type or *zpu1-204* homozygous kernels harvested 20 days

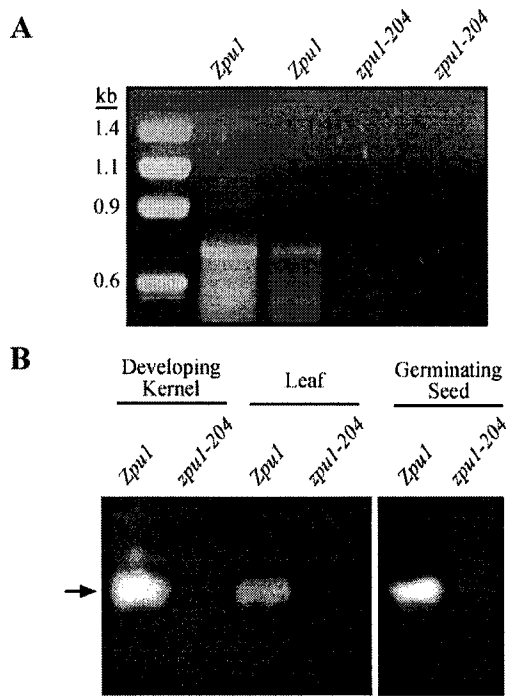

Figure 3.2. *zpu1-204* is a null allele. **A.** RT-PCR was performed on total RNA isolated from kernels at 20 DAP using the pulA/pulB primer pair. Two independent samples from wild-type Lanes 1 and 2) and *zpu1-204* mutants (Lanes 3 and 4) are shown. **B.** Zymogram analysis of wild-type *Zpu1* (W64A) and *zpu1-204* developing kernels, leaves, and germinating seeds. Pullulananse activity (indicated by the arrow) is visualized by clearing of the pullulan azure substrate. Approximately 50 μg of sample was loaded in all cases, except for *Zpu1* (W64A) germinating seed where 25 μg of protein was loaded to reduce the intensity of the band.

after pollination (DAP). RT-PCR performed using the pulA/pulB primer pair yielded a 0.7 kb fragment from the kernel RNA of both wild-type plants analyzed (Figure 3.2A), as expected from the sequence of the Zpu1 cDNA. The same procedure failed to amplify any *Zpu1*-specific fragment from kernels of two different *zpu1-204* mutant plants. Integrity of the total RNA samples from the wild-type and mutant plants was tested by ethidium bromide staining after agarose gel electrophoresis, and no differences were detected (data not shown). Thus, Zpu1 mRNA cannot be detected in *zpu1-204* mutants by this sensitive method.

Pullulanase-type DBE activity was undetectable in leaves, 20 DAP developing kernels, or germinating seeds of *zpu1-204* plants (Figure 3.2B). Proteins in total soluble extracts

were separated by native PAGE, then transferred to another polyacrylamide gel containing pullulan azure. This dyed polysaccharide polymer is a highly specific substrate of pullulanase-type DBE, so that enzyme activity is indicated by clear bands that result from cleavage of $\alpha(1\rightarrow6)$ linkages and diffusion of the maltotriose products out of the gel. Extracts from wild-type endosperm, leaves, and germinating seeds showed specific bands of pullulanase-type DBE activity in which the dyed substrate had been cleared. The same tissues from the *zpu1-204* mutant, however, all failed to show any such activity. Taken together, these data demonstrate that *zpu1-204* is a null mutation that conditions the complete elimination of pullulanase-type DBE.

Effects of ZPU1 Deficiency on Leaf Starch Metabolism

The *zpu1-204* mutation when homozygous does not condition any readily noticeable plant phenotype. Under field conditions, mutant plants grow at a normal rate following germination and show no obvious differences from the wild-type standard line in morphology or flowering time.

To specifically examine the effects of the *zpu1-204* on transient starch synthesis and degradation, leaf starch content was measured over a diurnal cycle of 16 h light, 8 h dark. Tissue samples were collected from adult maize leaves (approximately three weeks after planting, prior to anthesis) at various times in the cycle. The leaves were decolorized in boiling ethanol (80% [v/v]) and then stained with I_2/KI solution to reveal qualitatively the presence or absence of starch. The intensity of staining was similar in mutant and wild-type leaves at the end of the light period (Figure 3.3A), which indicates that starch accumulates to apparently normal levels in the absence of pullulanase-type DBE activity when plants are

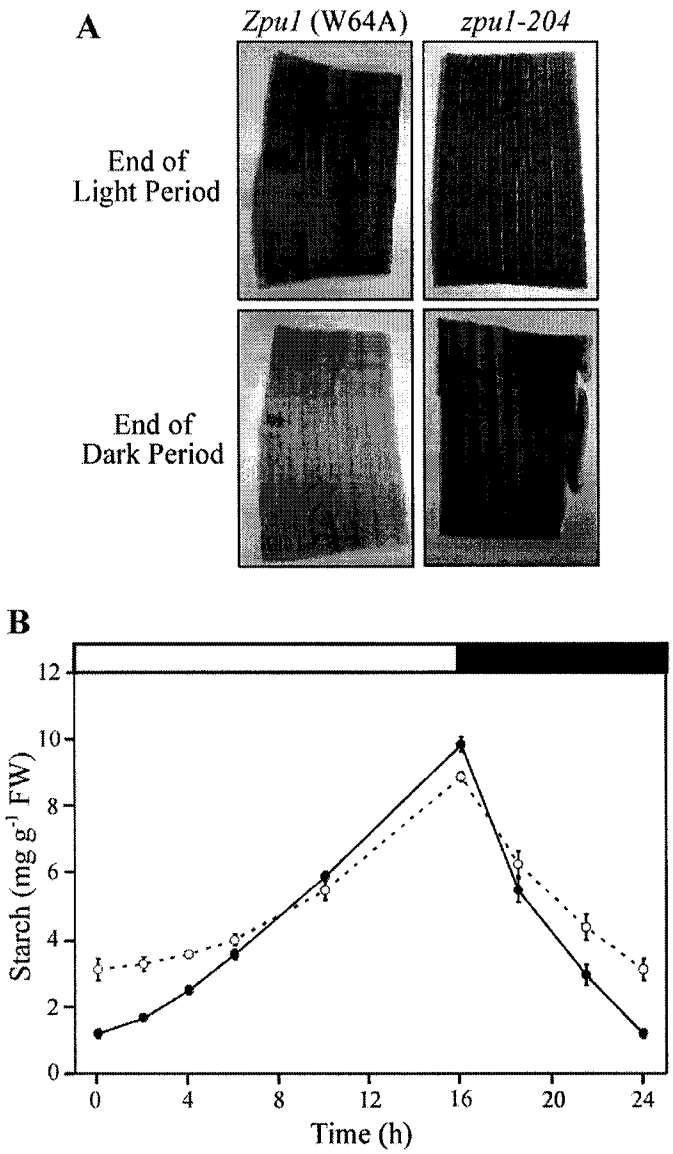

Figure 3.3. Leaf starch content during the diurnal cycle. **A.** Leaves from *Zpu1* (W64A) and *zpu1-204* mutants were harvested at the end of a 16 h light period and the end of the 8 h dark period, decolorized in boiling 80% (v/v) ethanol, and stained with I_2/KI solution. A qualitative measure of starch amount is indicated by the dark brown staining. **B.** Starch was extracted from leaves of individual plants and quantified by measuring the glucose released following complete digestion with amyloglucosidase. Closed symbols represent the wild-type and open symbols represent *zpu1-204*. Each point represents the mean ± SE of 15 plants. Where absent, error bars are smaller than the symbols.

grown under these conditions. As expected, at the end of the dark period the iodine stain failed to produce a deep brown color in wild-type leaves. The leaves of *zpu1-204* mutants, however, still stained brown at the end of the dark period (Figure 3.3A), indicative of a defect in starch catabolism. Thus, *zpu1-204* conditions a starch excess phenotype.

Quantification of leaf starch content during the diurnal cycle revealed that the rate of starch degradation is slower in *zpu1-204* plants (Figure 3.3B). The starch content exhibits a statistically significant reduction in *zpu1-204* leaves compared to wild-type (Student's two sample *t*-test, p-values <0.05) at 21.5 h and 24 h in the cycle. Removing plants from the diurnal cycle and extending the dark period to 16 h resulted in reduction of the starch level in *zpu1-204* leaves to the same level as wild-type (data not shown). Thus, pullulanase-type DBE affects the rate of starch degradation, but is not absolutely required for this metabolic process.

At the end of the light period the level of accumulated starch approaches that of wild-type leaves under normal diurnal conditions (Figure 3.3B, 16 h). Since the quantity of starch present at the beginning of the light period was greater in the mutant, the net amount of starch synthesis is reduced compared to wild-type. One possible explanation for these data is that the *zpu1-204* mutation causes a defect in starch accumulation. Alternatively, the reduced starch accumulation could be an indirect effect. For example, the Arabidopsis *dpe1* mutant (Critchley et al., 2001), which lacks disproportionating enzyme, is impaired in starch degradation and accumulates relatively large quantities of MOS during the night. Mutant plants subsequently synthesize less starch than the wild-type during the day. The authors speculate that the surplus starch and/or maltooligosaccharides (MOS) present at the end of

the dark period negatively influences synthesis in the following light period (Critchley et al., 2001).

To test for such carry-over effects from the preceding dark period, wild-type and *zpu1-204* plants were placed in the dark for 32 h to allow complete degradation of glucan. The destarched plants were then allowed to grow in the light for 16 h, after which time leaf starch was extracted, quantified, and analyzed with respect to starch composition and chain length distribution (see next section). Activity gel analysis shows that wild-type, destarched leaves possess normal levels of pullulanase activity compared to the untreated controls (data not shown). In these conditions, wild-type plants accumulated almost two-fold more starch than *zpu1-204* (Table 3.1). Plants bearing the *sbe2a::Mu* mutation (Blauth et al., 2001), which

Table 3.1. Carbohydrate Content of Destarched Leaves[a]

Genotype	Collection Time	Samples[b]	Metabolite (mg g^{-1} FW)		
			Starch	WSP[c]	Sucrose
Zpu1 (W64A)	End of Light Period	4	10.31 ± 0.16	0.84 ± 0.06	2.17 ± 0.28
zpu1-204	End of Light Period	4	5.23 ± 0.11	0.77 ± 0.15	4.59 ± 0.32
sbe2a::Mu	End of Light Period	2	6.35 ± 0.04	1.03 ± 0.18	5.52 ± 0.23
Zpu1 (W64A)	End of Dark Period	4	1.11 ± 0.03	0.19 ± 0.05	0.56 ± 0.10
zpu1-204	End of Dark Period	4	1.83 ± 0.03	0.26 ± 0.04	0.96 ± 0.07
sbe2a::Mu	End of Dark Period	2	1.19 ± 0.06	0.22 ± 0.04	1.36 ± 0.11

[a] Plants were destarched by extending the dark period to 32 h, transferred to the light for 16 h, followed by an 8 h dark period.
[b] Results are the mean ± SE of the number of samples indicated.
[c] Water soluble polysaccharide (WSP) is defined as the quantity of glucose equivalents liberated from polymers after treatment of total soluble extract with amyloglucosidase.

lacks the starch branching enzyme isoform SBEIIa, also showed a similarly reduced starch content. This analysis was necessary owing to the fact, shown in a following section, that *zpu1-204* conditions a reduction of SBEIIa activity as a secondary pleiotropic effect. Leaves of *zpu1-204* and *sbe2a::Mu* also show a relative increase in the amount of sucrose (Table 3.1), which is consistent with there being an altered partitioning between sucrose and starch as a result of the defect in starch synthesis.

Effects of ZPU1 Deficiency on Leaf Starch Structure

The composition of the leaf starch was analyzed by Sepharose CL2B chromatography. The amylopectin and apparent amylose content was essentially identical in granules from wild-type and the *zpu1-204* mutant (Figure 3.4). Starch of the *sbe2a::Mu* mutant in contrast, was markedly decreased in the relative amount of normal amylopectin and accordingly increased in lower molecular weight material that could be amylose or amylose together with low molecular weight amylopectin (Figure 3.4).

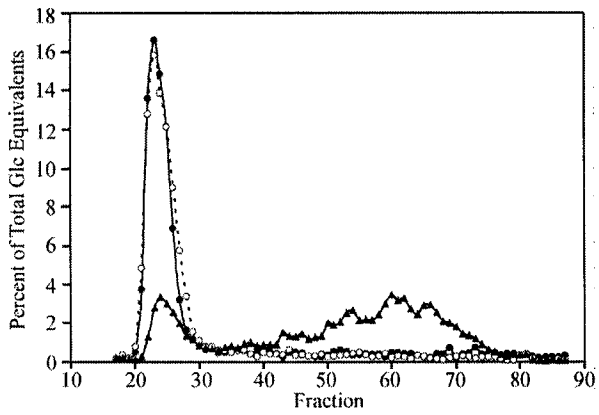

Figure 3.4. Sepharose CL2B separation of leaf starch. Starch was extracted from leaves of wild-type W64A (closed circles, solid line), *zpu1-204* (open circles, dashed line), and *sbe2a::Mu* (closed triangles, solid line) at the end of the photoperiod and applied to a 100-mL column. Fractions were eluted with 10 mM NaOH. Results were an average of two samples of each genotype. The data were normalized by dividing the amount of glucose equivalents in each fraction by the sum total of all fractions.

The structure of amylopectin in leaf starch was characterized regarding the distribution of linear chain lengths. Fractionated amylopectin from Sepharose CL2B chromatography was completely debranched by treatment with a bacterial isoamylase. Linear glucan chains in the resultant mixture were labeled with a fluorophore at the reducing end, separated by capillary electrophoresis, and quantified by fluorescence spectroscopy. Comparison of the distributions indicates that amylopectin from *zpu1-204* leaves contains significantly fewer smaller chains (8-15 DP [degree of polymerization]) than that from non-mutant leaves, and a slightly greater proportion of longer chains (Figure 3.5A, 3.5B). A similar amylopectin chain length profile was observed from *sbe2a::Mu* leaves (Figure 3.5C). Comparison between the profiles for *zpu1-204* and *sbe2a::Mu,* however, shows that *zpu1-204* has slightly more short chains (10-20 DP) than *sbe2a::Mu* (Figure 3.5D).

Effects of ZPU1 Deficiency on Endosperm Starch Metabolism

The content of glucose, sucrose, fructose, water soluble polysaccharide (WSP), and starch in endosperm tissue harvested 20 DAP was compared in isogenic *zpu1-204* plants and the non-mutant control line W64A (Table 3.2). The levels of sucrose and the monosaccharides, as well as the amount of starch, were statistically indistinguishable between mutant and wild-type endosperm. A statistically significant difference was observed, however, in the level of WSP, i.e., the glucan polymer present in the soluble phase after centrifugation at 10,000*g*, which was reduced in the mutant compared to the wild-type line. WSP from wild-type endosperm was characterized by capillary electrophoresis, and shown to consist of almost entirely of maltose, maltotriose, and maltotetraose (Figure 3.6, Intact). Treatment with a mixture of bacterial $\alpha(1\rightarrow6)$ glucan hydrolases failed to alter the

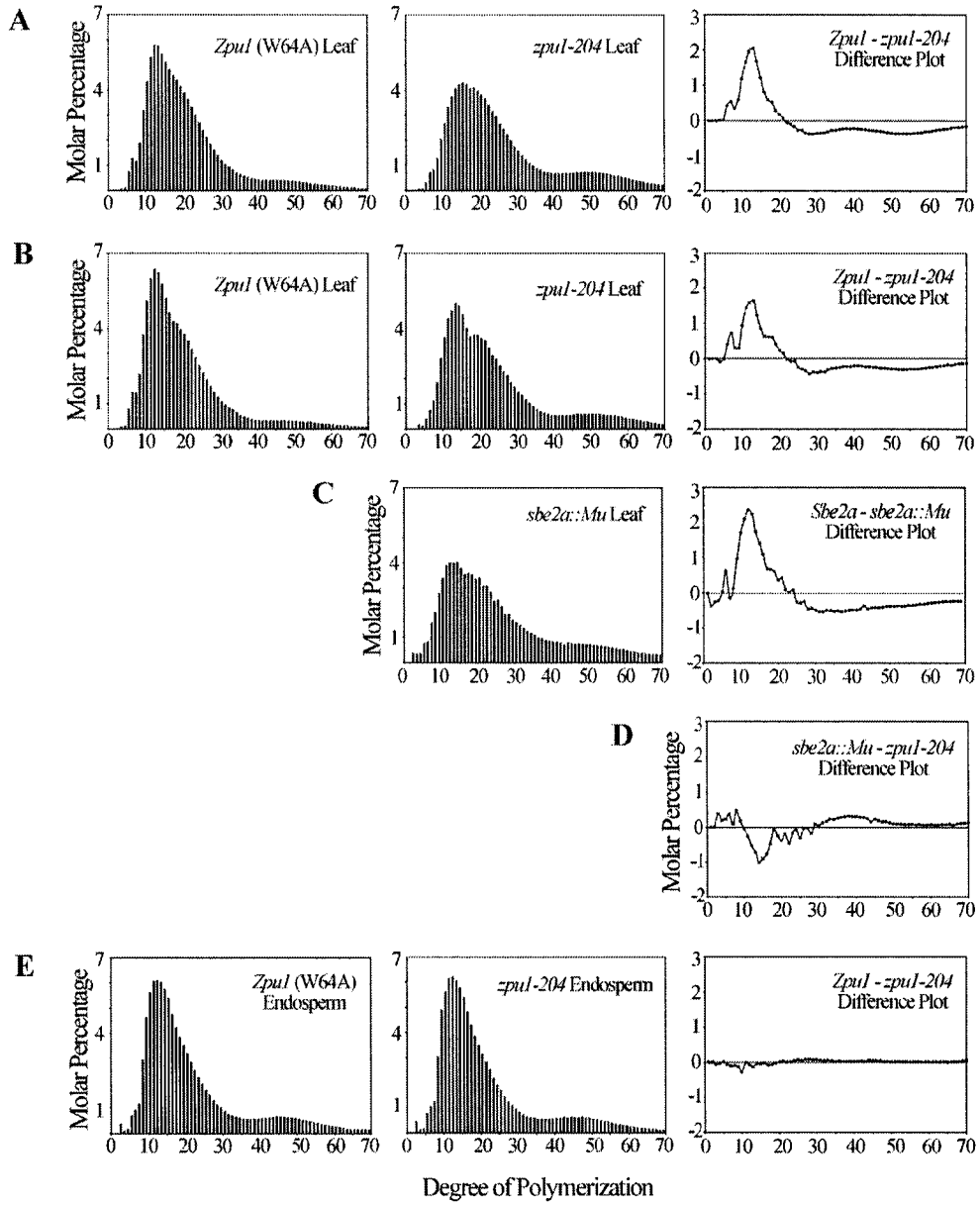

Figure 3.5. Chain length distributions of amylopectin. Amylopectin from pooled Sepharose CL2B chromatography fractions of endosperm and leaf starch was debranched with *Pseudomonas amyloderamosa* isoamylase. The reducing ends of the linear chains were labeled with the fluorophore APTS and separated by capillary electrophoresis. Individual chains are normalized to total peak area and differences in chain lengths are shown in subtraction plots. Results are the average of four independent analyses. Amylopectin from wild-type and *zpu1-204* leaves harvested at the end of the photoperiod from growth chamber-grown **A.** or field grown **B.** plants. **C.** Chain length distribution from *sbe2a::Mu* leaf amylopectin (compared to the same wild-type profile as in B). **D.** Difference plot comparing chain length distribution of *zpu1-204* and *sbe2a::Mu*. **E.** Amylopectin chain length distribution from wild-type and *zpu1-204* endosperm at 20 DAP.

electrophoretic profile, indicating that small branched polymers are not present in wild-type. In contrast, the WSP from *zpu1-204* endosperm contains significant quantities of low molecular weight, α(1→6)-linked polyglucans, as indicated by the increase following debranching in the abundance of chains comprising 5-8 glucose units (Figure 3.6). This is a distinctly different profile from that observed following debranching of phytoglycogen (Dinges et al., 2001), in which the most abundant chain lengths are 8-12 DP. These results suggest that ZPU1 functions during periods of endosperm starch accumulation to hydrolyze α(1→6) linkages in small, branched polysaccharides.

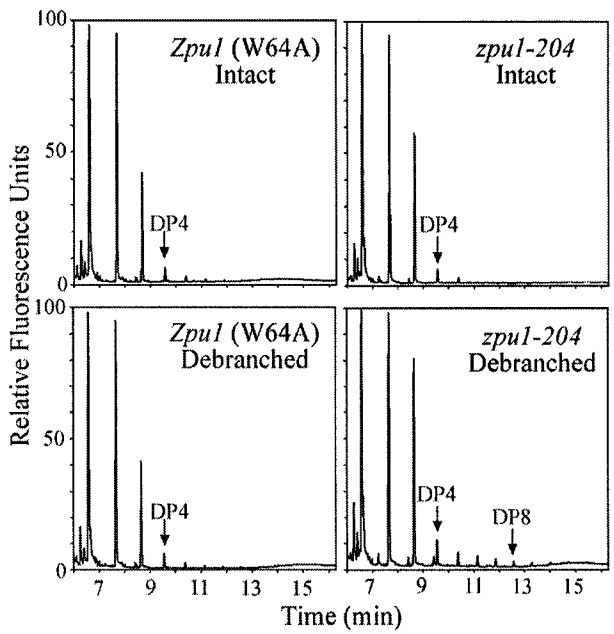

Figure 3.6. Analysis of WSP from *zpu1-204* endosperm. Water soluble glucan was labeled with APTS and subjected to capillary electrophoresis before and after treatment with a mixture of bacterial pullulanase and isoamylase. The profile of *Zpu1* WSP does not significantly change following debranching. The profile of *zpu1-204* WSP, however, shows a relative increase in chains greater than three glucose units, indicating the presence of small, branched polysaccharides. The degree of polymerization (DP) of each peak was assigned by comparing the retention time to known standards.

Table 3.2. Endosperm Carbohydrate Content

Metabolite	Genotype[a]				p-value[b]
	Zpu1 (W64A)		*zpu1-204*		(*Zpu1* vs.
	mg g^{-1} DW	%	mg g^{-1} DW	%	*zpu1-204*)
Glucose	4.48 ± 0.59	0.5	3.51 ± 0.40	0.4	0.07
Fructose	2.82 ± 0.29	0.3	2.01 ± 0.36	0.2	0.11
Sucrose	51.1 ± 2.80	6.1	48.7 ± 1.97	5.9	0.50
WSP	14.6 ± 0.83	1.7	9.97 ± 0.45	1.2	0.001
Starch	766 ± 31	91.3	768 ± 32	92.3	0.99

[a] Results are the mean ± SE of 12 total assays. Four ears of each genotype were harvested at 20 DAP, and three independent assays were performed on separate pools of kernels taken from each ear.

[b] Determined using Student's two sample *t*-test.

Endosperm starch composition and structure were compared in the wild-type and *zpu1-204* lines. The relative proportions of the amylose and amylopectin components of starch granules were similar, as determined by gel permeation chromatography on Sepharose CL2B (data not shown). The chain length distributions of purified amylopectin were determined for *zpu1-204* and wild-type endosperm, and essentially identical results were observed (Figure 3.5E). In endosperm tissue, therefore, seemingly normal starch accumulates to the wild-type level in the absence of any pullulanase-type DBE activity.

Effects of ZPU1 Deficiency on Starch Degradation During Germination

Pullulanase-type DBE is expressed *de novo* within the aleurone layer during germination of cereal grains, which suggests a role in degradation of storage starch. In

barley, for example, the same pullulanase-type DBE that is present in endosperm during development is also expressed during germination, and maximal mRNA accumulation is achieved at day 5 after planting (Longstaff and Bryce, 1993; Schroeder and MacGregor, 1998; Burton et al., 1999). Availability of the ZPU1-deficient maize mutant allowed a direct test of whether pullulanase-type DBE is required for kernel germination. Mature, dried wild-type and *zpu1-204* mutant kernels were hydrated in the dark, at 30°C. The time of cotyledon emergence was noted, and cotyledon length was recorded over successive days.

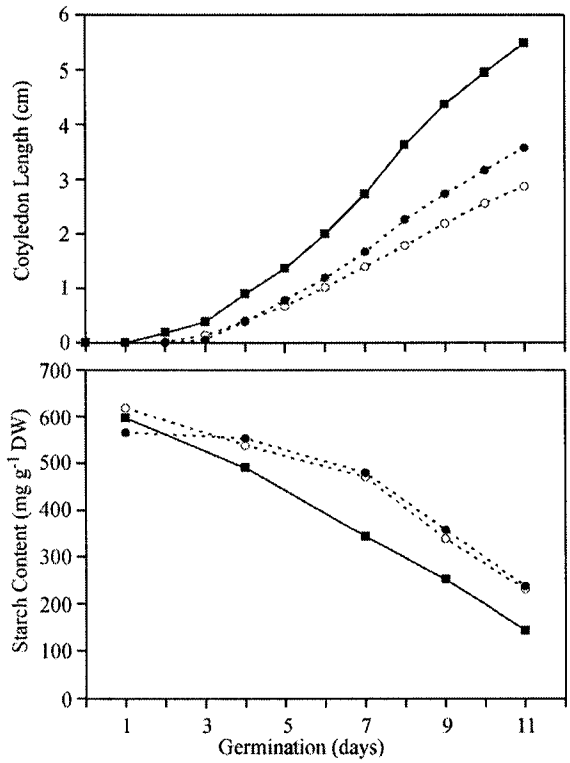

Figure 3.7. Germination analysis of *zpu1-204* kernels. Kernels from *Zpu1* (squares) and two different *zpu1-204* homozygotes (circles) were germinated on moist filter paper at 30°C. The lengths of the cotyledons were measured on successive days during the incubation period (Each point represents the mean of 15 cotyledons). Starch content in the germinating endosperm was determined by removing the roots and cotyledons from three kernels at each time point and measuring Glc equivalents of insoluble α-glucan polymer.

The percentage of mutant and wild-type kernels that germinated was nearly the same (data not shown). Cotyledons were first visible in the wild-type seeds 1-2 days earlier than in the mutants, and the average length of the mutant cotyledons was significantly less than that of the wild-type plants at all time points recorded over two weeks (Figure 3.7). This effect was obvious in kernels on the self-pollinated ears of two different *zpu1-204* homozygous plants. In the absence of ZPU1, therefore, seeds take longer to germinate and the rate of plant growth prior to attaining photoautotrophy is decreased compared to wild-type.

The amount of starch remaining in the endosperm of germinating kernels was measured periodically after cotyledon emergence, in order to test the hypothesis that retarded growth results from a reduced rate of starch degradation. Kernels from two different *zpu1-204* plants showed a slower rate of starch degradation until approximately day 7 of germination, compared to wild-type (Figure 3.7). After that time, the rate of starch degradation matched that of wild-type kernels. These results indicate that ZPU1 contributes to starch hydrolysis during the early stages of seed germination.

Effects of ZPU1 Deficiency on Other Starch Metabolizing Enzymes

In order to draw any conclusions about the direct effects of ZPU1 deficiency on starch metabolism, it is necessary to determine whether other biosynthetic or degradative enzymes are affected pleiotropically by *zpu1-204*. This is particularly relevant to DBEs in rice and maize, because pleiotropic effects on both SBE and pullulanase-type DBE are known to result from mutations affecting the isoamylase-type DBE SU1 (Beatty et al., 1999; Kubo et al., 1999; Dinges et al., 2001). Zymogram assays were applied to broadly characterize the

starch-metabolizing activities present in total soluble extracts from wild-type and *zpu1-204* mutant kernels harvested 20 DAP, as well as wild-type and mutant leaves collected at the end of the light or dark phase of the standard diurnal cycle. Proteins were separated by native PAGE, then electrophoretically transferred to a gel containing 0.3% (w/v) potato starch. Staining of the gels after incubation results in series of colored bands indicative of specific enzyme activities (Figure 3.8A), many of which have been identified using mutant analysis, partial purification, and immunological characterization (unpublished results).

The *zpu1-204* mutation clearly has a secondary effect on the activity of the branching enzyme isoform SBEIIa, which was undetectable in kernel extracts and significantly reduced in leaf and germinating seed extracts of mutant plants (Figure 3.8A). A pleiotropic effect also was observed on an amylolytic activity presumed to be a β-amylase (Figure 3.8A). A novel β-amylase activity band was observed in ZPU1-deficient kernels, and the intensity of the normal band appears to be slightly reduced compared to wild-type in endosperm and light-harvested leaves (Figure 3.8A). No defects were observed in other starch metabolizing enzymes, particularly isoamylase-type DBE, SBEIIb, or SBEI (Figure 3.8A). Isoamylase-type DBEs were characterized in further detail in order to reveal the major activity band that is obscured by SBEI. After fractionation of crude extracts by anion exchange chromatography, zymogram analysis showed the presence of three characteristic blue bands in extracts from both wild-type and *zpu1-204* kernels (data not shown). These data confirm that loss of pullulanase-type DBE does not pleiotropically affect the isoamylase-type DBE. A separate zymogram analysis was performed to observe starch synthase activities. Four SS activity bands were apparent in both *zpu1-204* and wild-type W64A leaf extracts from tissue

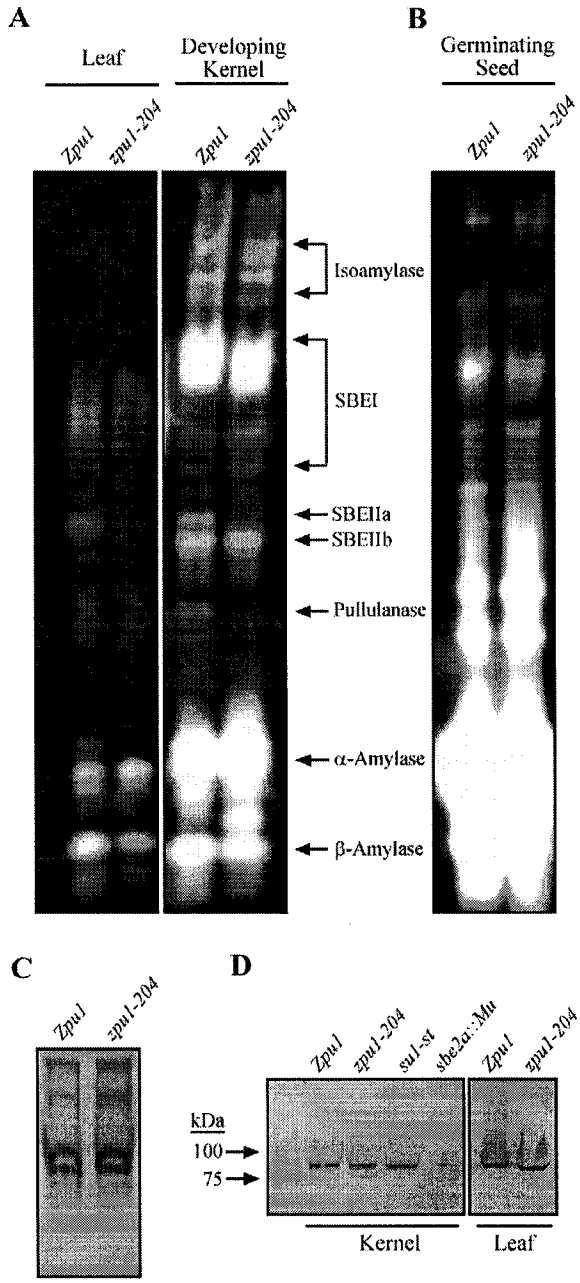

Figure 3.8. Activity gel analysis of starch modifying enzymes. Approximately 100 μg total protein extracts from wild-type W64A and *zpu1-204* leaves **(A)**, 20 DAP kernels **(A)**, and germinating seeds **(B)** was separated on a 15 cm native polyacrylamide gel, then electroblotted to a starch-containing gel. Starch modifying enzyme activities are visualized by staining with I_2/KI. **C.** To detect starch synthase activities, total leaf extracts were separated in a native gel containing 0.2% glycogen, incubated 16 h in the presence of ADP-Glc and stained with iodine. **D.** Immunoblot from SDS-PAGE of total protein extracts developed with antisera against SBEIIa.

harvested at the end of the light phase of the standard diurnal cycle, and no differences were discernable between the two lines (Figure 3.8C).

Immunoblot analysis was used to determine whether the lack of SBEIIa activity in *zpu1-204* leaves and kernels results from failure to accumulate the protein. Polypeptides in total soluble extracts were separated by SDS-PAGE, and then probed with a polyclonal antibody that recognizes SBEIIa (Figure 3.8D). The antibody detects a protein of the expected molecular weight, approximately 85 kD, which is not present in a control *sbe2a::Mu* mutant line (Blauth et al., 2001). Furthermore, the protein is present in approximately equal abundance in both the wild-type and *zpu1-204* plants, in both developing kernel and leaf tissue. The *su1-st* mutation, in the gene coding for the major isoamylase-type DBE, has the same pleiotropic result of eliminating SBEIIa activity (Dinges et al., 2001). In this mutant, the SBEIIa protein also accumulates to the same level as wild-type. Secondary effects on SBEIIa activity, therefore, are exerted at the post-translational level by mutation of either the pullulanase-type DBE ZPU1 or the isoamylase-type DBE SU1.

Water-soluble Polysaccharide Accumulation Conditioned by *zpu1-204*

A null mutation in the *su1* gene, which codes for an isoamylase-type DBE, conditions a reduction of 80% in the amount of starch produced in endosperm tissue, and also the appearance of significant quantities of phytoglycogen. The reason that some starch granules form even in the complete absence of SU1 is not clear. Activity provided by the *Iso2* and *Iso3* genes may account for the residual starch, but there is no direct evidence that these enzymes contribute to starch metabolism in maize endosperm. Another plausible explanation

is that the pullulanase-type enzyme provides some of the DBE function required for normal starch accumulation. Double mutants containing both *zpu1-204* and a *su1* mutation were constructed in order to test this hypothesis, with the expectation that overlapping function of the two DBEs would result in a more severe starch biosynthesis defect than that conditioned by either mutation alone. The allele *su1-st* (Dahlstrom and Lonnquist, 1964; Dinges et al., 2001) was utilized because it causes only a modest reduction in the amount of starch, accumulation of relatively small amounts of phytoglycogen, and a mild kernel phenotype

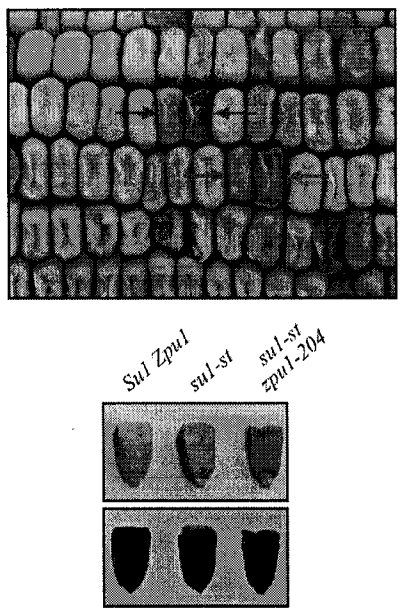

Figure 3.9. Synthetic phenotypes of DBE double mutants. Crosses were made between *zpu1-204* and the isoamylase-type DBE mutant *su1-st*. A portion of an ear resulting from the self-pollination of the double heterozygote is shown. Approximately three-sixteenths of the kernels possess the *su1-st* phenotype (blue arrows), having a slightly wrinkled, translucent crown, but an opaque, starchy central and basal region (observed in the side view of the kernels, as seen with overhead light and on a lightbox). Kernels possessing a markedly more severe phenotype appeared in a ratio of approximately one-sixteenth (green arrows). These kernels are characterized by a more severe wrinkling of the crown, extending further into the central region of the kernel compared to *su1-st* homozygotes. PCR genotyping of these kernels indicates that they are homozygous for the *zpu1-204* allele, but singly homozygous ears for this allele show no phenotype.

compared to the severely shrunken and translucent kernels that result from the *su1* null mutation. In this genetic background, a further defect in the process that accounts for phytoglycogen accumulation and granular starch deficiency would be readily detectable.

Plants homozygous for *zpu1-204* were crossed to a homozygous *su1-st* line, and the resulting double heterozygotes were self-pollinated. Three distinct kernel phenotypes were distinguishable on the F_2 ears from this cross (Figure 3.9). In addition to kernels with wild-type appearance, the moderate but clearly recognizable kernel phenotype conditioned by *su1-st* was present in approximately three-sixteenths of the progeny. A third distinct phenotype, obviously more severe than the *su1-st* single mutant, appeared at the approximate frequency of one-sixteenth, and these kernels were expected to be the *zpu1-204; su1-st* double mutants. Like *zpu1-204*, *su1-st* can be detected definitively by PCR analysis (Dinges et al., 2001). This technique was applied to DNA of 45 seedlings grown from severely mutant kernels. The PCR data in all instances confirmed that these individuals were *zpu1-204; su1-st* double homozygotes. Thus the ears derived from self pollination of the double heterozygote allow comparison between sibling kernels, either wild-type (*Su1/Su1* or *Su1/su1-st*; *Zpu1/Zpu1*, *Zpu1/zpu1-204*, or *zpu1-204/zpu1-204*), *su1-st* single mutant (*su1-st/su1-st*; *Zpu1/Zpu1* or *Zpu1/zpu1-204*), or double mutant (*su1-st/su1-st*; *zpu1-204/zpu1-204*).

Single mutants homozygous for *su1-st* display slight wrinkling on the crown of the kernel, and the *zpu1-204* homozygous mutant kernels, as mentioned previously, appear to be identical to wild-type. The double mutants are easily distinguished from either single mutant by severe wrinkling at the crown and a fully translucent appearance on the ear (Figure 3.9). Such an appearance typically is indicative of significant amounts of phytoglycogen in the

affected kernels. Side views of kernels removed from the ear showed that the extent of the apparent phytoglycogen-containing region was much greater in the double mutant kernels than in the *su1-st* single mutants (Figure 3.9). Carbohydrates were extracted from kernels of the different phenotypes and starch and WSP were quantified (Table 3.3). The double mutant kernels contained five times the WSP quantity of the *su1-st* single mutants, and were reduced by 15% in the content of granular starch. Like phytoglycogen, the WSP from *su1-st; zpu1-204* double mutants was precipitable in 5 volumes of 100% [v/v] ethanol. From these results we conclude that ZPU1 is capable of participating in the process of starch biosynthesis, by providing some functional overlap with that of the isoamylase-type DBE

Table 3.3. Endosperm Carbohydrate Content in DBE Double Mutants

Metabolite	Phenotype					
	Wild-type Segregants		*su1-st*		*su1-st; zpu1-204*	
	mg g^{-1} DW	%	mg g^{-1} DW	%	mg g^{-1} DW	%
Glucose	9.4 ± 0.4	1.3	20.6 ± 3.0	2.8	43.9 ± 9.0	4.7
Fructose	3.7 ± 0.5	0.5	5.5 ± 1.3	0.8	8.6 ± 1.4	0.9
Sucrose	4.0 ± 0.6	0.5	5.7 ± 0.6	0.8	16.5 ± 3.7	1.8
WSP	12.3 ± 3.1	1.7	56.1 ± 10.9	7.7	310 ± 44	33
Starch	704 ± 49	96	646 ± 43	88	549 ± 21.5	59

Kernels resulting from the self-pollination of *Su1/su1-st; Zpu1/zpu1-204* plants were selected based upon their phenotype. Segregants with a wild-type phenotype may have the genotype *Zpu1* or *zpu1-204*. Five mature, imbibed kernels were pooled for each assay. Measurements are the mean ± SE of four independent assays.

SU1 and/or contributing to the total amount of α(1→6) hydrolytic activity in the developing endosperm. This is despite the fact that ZPU1's biosynthetic function is not essential for production of normal quantities of endosperm starch or amylopectin with the wild-type distribution of linear glucan chain lengths.

Starch granule size and number in DBE mutants

Mutational analysis has shown that a defect in an isoamylase-type DBE of maize and barley causes an increase in the number of starch granules in endosperm tissue compared to wild-type (Creech, 1968; Burton et al., 2002). The mutant granules are compound in nature, and are smaller than those produced in wild-type endosperm (Creech, 1968; Burton et al., 2002). These observations led to the hypothesis that the isoamylase-type DBE is important for proper starch granule initiation (Burton et al., 2002), functioning as a negative factor. To

Table 3.4. Starch granule characteristics of DBE mutants.

Sample	Granules per endosperm[a] (millions)	Granule size distribution (μm)[b]		
		10%	50%	90%
Zpu1 (W64A)	9.90 ± 0.27	1.88	5.29	12.3
zpu1-204	10.02 ± 0.25	2.06	5.08	11.5
su1-st	17.14 ± 1.18	1.64	2.86	6.25
su1-st; zpu1-204	40.51 ± 2.71	1.57	2.42	5.62

[a] The endosperm starch of three kernels harvested 20 DAP was extracted together. Results are the mean ± SE of three replicate aliquots of the starch suspension. Each aliquot was sampled six times and counted using a hemacytometer.

[b] Determined using a Coulter Counter.

examine whether pullulanase-type DBE affects granule initiation, starch granules extracted from endosperm at mid-development (20 DAP) were counted using a hemacytometer (Shannon et al., 1996) and their size distribution was measured using a Coulter Counter. Wild-type and *zpu1-204* endosperm contained a similar number of granules per kernel (Table 3.4), and their size distribution was similar. From these data there is no suggestion that ZPU1 functions as negative regulator of granule initiation.

An effect of ZPU1 deficiency on granule number was observed, however, when isoamylase-type DBE function was also compromised. As expected, *su1-st* conditioned a significant increase in the number of granules per kernel compared to wild-type, and these granules were smaller than normal (Table 3.4). The *su1-st*; *zpu1-204* double mutant showed a further increase in granule number per kernel, approximately two-fold more than the *su1-st* single mutant (Table 3.4). Therefore, ZPU1 is capable of functioning as a factor that determines granule number, and again the two different types of DBE appear to overlap functionally in the process of starch biosynthesis.

Discussion

In order to investigate the function(s) of the pullulanase-type DBE in starch metabolism, a reverse genetics approach was used to identify transposable element insertions in the maize *Zpu1* gene. The mutation *zpu1-204* was shown to be a null allele by analysis of the gene structure and by the complete lack of detectable mRNA transcript or enzymatic activity. These results demonstrate that the protein coded for by *Zpu1* provides the only pullulanase-type DBE activity in maize, and they are consistent with the fact that the gene coding for this enzyme is unique (Beatty et al., 1999). Despite the complete loss of pullulanase-type DBE

activity, plants homozygous for *zpu1-204* do not exhibit any obvious morphological differences in the leaf or kernel. This fact accounts for the lack of mutations in pullulanase-type DBE detected in any forward genetic screen. Application of reverse genetics, however, revealed that loss of ZPU1 activity alters various aspects of starch metabolism.

Pullulanase-type DBE is required for normal starch catabolism. Germinating mutant seeds show a decreased rate of starch degradation and a slower rate of cotyledon growth compared to wild-type. This effect occurs in the presence of very high levels of amylolytic activity and apparently normal levels of isoamylase-type DBE (Figure 3.8B). Leaves of *zpu1-204* also exhibit a slower rate of starch catabolism, as indicated by an increased amount of granular starch at the end of the dark period compared to wild-type. This effect is observed in leaves operating under a diurnal cycle (Figure 3.3B) and in destarched leaves (Table 1). One possible explanation is that the total level of $\alpha(1\rightarrow6)$ glucosidase activity, i.e. both pullulanase-type and isoamylase-type DBE, may be limiting in germinating *zpu1-204* seeds and leaves. Alternatively, pullulanase-type DBE might serve a specific catalytic function in starch catabolism that cannot be substituted by any other glucan hydrolases. Accordingly, isoamylase-type DBE would not fully compensate for loss of pullulanase-type DBE. *In vitro* studies of recombinant maize pullulanase-type DBE show clear differences in substrate preference compared to isoamylase-type DBE, with pullulanases generally preferring shorter chains (Rahman et al., 1998; Wu et al., 2002). This fact, along with the high degree of evolutionary conservation of both classes of DBEs, is consistent with the hypothesis that enzyme specificity is the reason that starch degradation is reduced in *zpu1-204* mutants.

The observed reduction in starch degradation raises the possibility that owing to altered carbon partitioning there could be a deficiency in general plant growth characteristics. Obvious qualitative defects were not observed in kernel size, ear development, plant height, etc., although further characterization is required to determine whether any quantitative defects on traits such as yield might result from mutations affecting the *zpu1* locus.

In addition to the demonstrated and anticipated role of ZPU1 in starch degradation, this study revealed that the pullulanase-type DBE also is capable of functioning in starch anabolism. Expression of pullulanase-type DBE in the developing seeds of many plant species, such as maize (Beatty et al., 1999), rice (Nakamura et al., 1996a), and pea (Zhu et al., 1998) is consistent with a role during starch synthesis. Evidence that pullulanase-type DBE may be involved in starch synthesis is shown here by the fact that in some genetic backgrounds *zpu1-204* can condition a "sugary" kernel phenotype. This condition includes an increase in starch granule number, reduced amount of granular starch, and significant amounts of phytoglycogen in developing endosperm. Pleiotropic effects on SBEIIa activity can be ruled out as the causative agent of this phenotype in the *su1-st* background, because *su1-st* itself conditions loss of SBEIIa activity. Thus we conclude that the enhanced starch biosynthesis defect observed in the *su1-st; zpu1-204* double mutant is a specific effect of the loss of ZPU1.

This effect of *zpu1-204* was observed only in the presence of a mutation affecting isoamylase-type DBE, not in the wild-type genetic background. The two classes of DBE, therefore, appear to overlap to some extent in their biosynthetic function despite the fact that each enzyme class has been separately conserved throughout the evolution of bacteria and plants. The simplest explanation is that ZPU1 functions during the normal starch

biosynthesis process, however, from the available data the possibility cannot be excluded that this function arises only in the abnormal conditions engendered by alteration of isoamylase-type DBE. The specific function of pullulanase-type DBE in the isoamylase-compromised background is not yet known. Both direct and indirect functions are consistent with the data presented here, including pre-amylopectin processing, a role in the metabolism of maltooligosaccharides (MOS), or involvement in starch granule initiation.

Previous analysis of DBE activities in isoamylase-type DBE mutants of rice indicated a correlation between regions of the endosperm that accumulated significant amounts of pullulanase-type DBE activity and the presence of starch granules in those particular cells (Nakamura et al., 1997). From these data the hypothesis was suggested that pullulanase-type DBE, not isoamylase-type DBE, may be the primary determinant of whether or not phytoglycogen accumulates. This possibility can now be excluded based on the fact that *zpu1-204* endosperm tissue exhibits normal starch structure and quantity in the absence of any pullulanase-type DBE activity. Rather, the principal factor appears to be the combined activity of the two DBEs. A critical level of isoamylase-type DBE activity is clearly required to prevent accumulation of phytoglycogen. When this enzyme is defective, pullulanase-type DBE may become limiting in determining the amount of phytoglycogen versus granular starch produced. The incomplete ability of ZPU1 to fully compensate for loss of the isoamylase-type DBE may be explained by limitation of ZPU1 to hydrolyze select substrates. This interpretation is entirely consistent with the results of the study in rice (Nakamura et al., 1997), because both isoamylase- and pullulanase-type DBE are affected by the *su1*- mutations in the lines that were analyzed.

In genetic backgrounds with intact isoamylase-type DBE, pullulanase-type DBE still performs a metabolic function. Small amounts of free glucan polymer in the form of WSP are present in developing wild-type endosperm during the period of starch accumulation (Figure 3.6), and the concentration of this material is reduced to a statistically significant extent as a result of *zpu1-204* (Table 3.2). Pullulanase-type DBE activity, therefore, appears to function to produce MOS through hydrolysis of branch linkages in a precursor polymer. The source of the precursor polymer that gives rise to the MOS is not known. One possibility is that trimming of pre-amylopectin by DBEs produces these polymers (Ball et al., 1996; Myers et al., 2000). A population of glucan chains at the surface of the granule, not present in mature starch, has recently been shown to exist transiently in Arabidopsis leaves (Nielsen et al., 2002). If similar structures were to exist in developing maize endosperm, ZPU1 could be involved in cleaving some of these chains to generate MOS.

Within the soluble material in the mutant there is a detectable quantity of $\alpha(1\rightarrow6)$-branched polymer with chains ranging from DP3 to DP10, which is not present in wild-type (Figure 6). This observation is consistent with the suggestion that branched oligosaccharides exist transiently in the soluble phase and are cleared by degradative activities including $\alpha(1\rightarrow6)$ glucan hydrolase (Zeeman et al., 1998b). Some of the necessary hydrolytic activities are likely specific to pullulanase-type DBE, because isoamylase-type DBE is present in *zpu1-204* endosperm at apparently normal levels and yet the branched soluble polymer still accumulates.

Starch biosynthesis is also clearly affected in the leaves of the *zpu1-204* mutant, as indicated by the altered amylopectin structure and reduced accumulation of starch in the light phase (Figure 5A, 5B, Table 1). These effects might both be explained by a direct role for

the ZPU1 protein in this process or, instead, by one or more pleiotropic effects. In particular, the pleiotropic effect of *zpu1-204* on SBEIIa activity must be considered. Loss of SBEIIa, which is a major form of branching enzyme in the maize leaf, is known to affect the composition and structure of starch (Blauth et al., 2001 and Figure 4, 5C). The effects of *zpu1-204* on chain length distribution of leaf amylopectin are similar to, but not as severe as those of the *sbe2a::Mu* mutation (Figure 3.5D). These effects are not universal, because amylopectin structure in endosperm starch is not affected by *zpu1-204*. This could be explained by differences in the isoform components of the biosynthetic systems, i.e., SSs and SBEs, in these two tissues. Thus, while the *zpu1-204* mutation clearly affects leaf starch synthesis, it cannot be concluded that this effect is due to loss of ZPU1 directly.

There are distinct differences, however, in the effects of *zpu1-204* or *sbe2a::Mu* on chain length distribution, which might not be expected given the fact that SBEIIa activity is reduced in both instances. Furthermore, the *sbe2a::Mu* mutation has severe effects on starch composition (Figure 3.4), where *zpu1-204* does not. Possibly, lack of pullulanase-type DBE causes an altered starch structure by itself, which is distinct from that caused by the SBEIIa deficiency. Alternatively, the effect that the *zpu1-204* mutation has on SBEIIa, in which an apparently inactive polypeptide is present, might provide for a different amylopectin structure than that obtained when there is a complete lack of SBEIIa protein.

Analysis of *zpu1-204* mutant plants provides additional examples of the numerous pleiotropic interactions occurring among starch biosynthetic enzymes, which seem to involve particularly the DBEs. In maize and rice, molecular defects in isoamylase-type DBE have long been known to secondarily reduce the activity the pullulanase-type DBE (Pan and Nelson, 1984; Nakamura et al., 1996b). The converse effect, in which loss of

pullulanase-type DBE negatively affects the activity of SU1, does not appear to occur. Loss of ZPU1, however, does cause a defect in activity of SBEIIa in leaves and endosperm. The same deficiency in SBEIIa also is a secondary effect of a specific mutation in the isoamylase-type DBE, which provides further indirect evidence in support of functional interaction between the two types of DBE. Transcriptional or translational regulation does not account for the effects on SBEIIa, as the polypeptide accumulates to seemingly normal levels in both the *zpu1-204* and *su1-st* mutants. Multisubunit complexes must be considered as a possible explanation for allele-specific defects of this type, although little direct information is currently available.

The metabolic processes of starch catabolism and anabolism require that the plant evaluate environmental, temporal and developmental cues, and then regulate an entire suite of enzymes based upon the information contained in those signals. This investigation of the *zpu1-204* mutation has shown that some enzymes of starch metabolism may have a dual function, being required for periods of both net synthesis and degradation. In addition to the starch phenotypes described here, the specific pleiotropic effects of *zpu1-204* on both a starch biosynthetic enzyme (SBEIIa) and a degradative enzyme (β-amylase) indicate that ZPU1 interacts with components of both the assembly and disassembly machineries. Only a small part of the regulation of starch synthesis and degradation involves the control of gene expression at the transcriptional or translational level. Rather, the transitions between the processes of synthesis and degradation are likely to involve more subtle and responsive changes to signaling mechanisms, perhaps mediated by altered direct interactions between enzymes that recognize glucose homopolymers as substrates.

Materials and Methods

Genetic Analysis and Generation of Plant Material

Plants used for biochemical and molecular analyses were of the BC_3S_2 generation, which was produced as follows. TUSC F_1 maize (*Zea mays* L.) seeds were generated by Pioneer Hi-Bred International, Inc. (Johnston, IA) by crossing a *Mu*-active line (i.e. possessing a high *Mutator* copy number and the autonomous element, *MuDR*) to a non-*Mutator* line. PCR screening of DNA pools (see following section) was performed on DNA isolated from the leaves of the F_1 plants and they were self-pollinated to give F_2 seed. The F_2 seed was provided by Pioneer, and PCR analysis of leaf DNA samples from progeny plants identified *Zpu1/zpu1* heterozygotes. These were crossed to inbred line W64A, and identification of heterozygotes by PCR and crossing to W64A was repeated three additional times to generate BC_3 ears. A heterozygote grown from BC_3 seed was self-pollinated and seeds from the resulting BC_3S_1 ear were planted. Homozygous *zpu1-204* plants (representing one-fourth of the progeny) were identified by PCR analysis. Self-pollination of those plants generated the BC_3S_2 kernels used for analysis of endosperm tissue, and plants grown from those kernels were used for analysis of leaf tissue. The wild-type controls for both leaves and developing endosperm were from inbred W64A plants. Immature kernels or leaf samples were quick-frozen in liquid nitrogen and stored at -80°C until further analysis.

Plants used for analysis of leaf starch were grown in a growth chamber in a diurnal cycle comprising alternating periods of 8 h darkness at 25°C - 16 h light at 28°C with approximately 280 μmol quanta m^{-2} s^{-1} of mixed fluorescent and incandescent light. The sixth, seventh, eighth, and ninth vegetative leaves (V6-V9) were harvested approximately 3

weeks after planting for analysis of starch structure and content. Other plants were grown under field conditions in Ames, IA or Molokai, HI.

The *su1-st* mutation was characterized in detail in a previous report (Dinges et al., 2001). All *su1-st* lines used in this study were derived from crosses to the W64A inbred for a minimum of five generations. The *sbe2a::Mu* mutation was described in a previous report (Blauth et al., 2001). Adult leaves (post-anthesis) from greenhouse grown plants homozygous for *sbe2a::Mu* were provided by Dr. Mark Guilitinan (Pennsylvania State University, University Park). The *sbe2a::Mu* mutation originally was reported to co-segregate with a leaf-senescence phenotype (Blauth et al., 2001). After repeated backcrossing, this phenotype was separated from *sbe2a::Mu*, and the mutant leaves lacking SBEIIa appear normal (Blauth et al., 2002).

Genotype Determination and Gene Expression Analysis by PCR

DNA was isolated from fresh or lyophilized maize leaves using CTAB extraction (Saghai-Maroof et al., 1984). Total RNA was isolated from endosperm harvested 20 DAP using the TRIzol Reagent (Catalog No. 15596-026, Invitrogen, Carlsbad, CA) according to the supplier's instructions. TUSC pool screening was performed by Pioneer Hi-Bred International, Inc. (Bensen et al., 1995). One of the oligonucleotide primers was taken from the Zpu1 cDNA sequence, either pulA (5'-AAT CCA AAC GCG GAC GCA AAT GTT GCT C-3') (nt 22-50 on the forward strand) or pulB (5'-ACT TAC ATC CTG TGC TGT AGG AGC CCA-3') (nt 727-701 on the reverse strand). The second PCR primer in each reaction was the *Mu* terminal inverted repeat sequence 9242 (5'-AGA GAA GCC AAC GCC A(AT)C GCC TC(CT) ATT TCG TC-3').

The primers pulA, pulB, and 9242 also were used for genotyping of individual plants. The parameters of these PCR reactions were 95°C for 3 min followed by 35 cycles of 95°C for 1 min and 68°C for 2 min. The reactions were performed in the presence of 5% DMSO (v/v), using Platinum *Taq* polymerase (Catalog No. 11304-029, Invitrogen) in the buffer conditions suggested by the supplier with a final $MgCl_2$ concentration of 3.5 mM. RT-PCR amplification endosperm RNA was performed using the Superscript One-Step RT-PCR system (Catalog No. 10928-034, Invitrogen) and the pulA/pulB primer pair, according to the manufacturer's instructions.

Carbohydrate Extraction and Quantification

Carbohydrate extraction from developing kernels harvested 20 DAP, and quantification of glucose, fructose, sucrose, granular starch, and WSP were performed as described previously (Dinges et al., 2001). Mature, dried kernels were analyzed by the same procedure, except that they were first soaked overnight at 50°C in 0.3% (w/v) sodium bisulfite, and pericarp and embryo were then removed by manual dissection. Leaf starch content over a diurnal cycle was determined according to a previously described procedure (Zeeman et al., 1998a), with slight modification. Approximately 500 mg of leaf tissue was harvested, weighed, and immediately boiled in 50 mL of 80% (v/v) ethanol. The decolorized leaves were then homogenized in 80% (v/v) ethanol using a mortar and pestle. The homogenate was centrifuged at 1,500*g* for 10 min at room temperature, and the pellet was extracted again with 80% (v/v) ethanol. The pelleted material was suspended in 4 mL water and boiled for 30 min. Total α-glucan polysaccharide, presumed to be nearly entirely derived from granular starch, was quantified using a commercial assay kit that measures glucose released

following digestion with amyloglucosidase (Catalog No. E0207748, R-Biopharm, Darmstadt, Germany).

To isolate leaf starch for structural analysis, five grams of tissue were ground in a mortar and pestle in 500 mM 3-(N-morpholino) propane sulfonic acid (MOPS), pH 7.5, 5 mM EDTA, 10% ethylene glycol. The homogenate was filtered through Miracloth and centrifuged at 5,000*g* for 10 min. The supernatant was boiled and stored at -20°C. The pellet was suspended in 1 mL extraction buffer and 9 mL Percoll (Catalog No. 17-0891-02, Amersham Biosciences, Piscataway, NJ). The Percoll gradient established by centrifugation at 10,000*g* for 30 min yielded a starch pellet largely free of other cell components. Starch granules were solubilized by boiling in 90% (v/v) DMSO for 30 min, then stored at -20°C until further analysis.

The size distribution of isolated starch grains was estimated using a Coulter Counter (Multisizer II, Beckman-Coulter) equipped with 70 μm aperture. The starch pellet was thoroughly suspended in water. An aliquot was added to the diluent and immediately placed in the instrument.

Amylopectin Structural Analysis

Amylopectin and amylose in the granular starch fraction were separated by gel permeation chromatography as described previously (Dinges et al., 2001). Chain length distribution was determined by fluorophore-assisted carbohydrate electrophoresis (FACE), following a previously published procedure (O'Shea et al., 1998) with slight modification. The lyophilized amylopectin fraction was suspended at 4 mg glucose equivalents mL^{-1} in 30% DMSO, then boiled for 10 min. A 10 μL aliquot, containing 200 μg glucose equivalents,

was diluted to a final volume of 50 μL with 50 mM sodium acetate, pH 4.5. *Pseudomonas* sp. isoamylase (1 μL, 0.3 units) (Catalog No. E-ISAMY, Megazyme International, Bray, Ireland) was added, and the reaction was incubated overnight at 42°C. The mixture was heated in boiling water for 5 min, then centrifuged for 2 min at full speed in a microfuge. A 10 μL sample of the reaction, containing 4 μg glucose equivalents, was evaporated to dryness in a Speed Vac.

The reducing ends of the liberated oligosaccharide chains were derivatized with the fluorescent compound 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) (Catalog No. 09341, Sigma-Aldrich, St. Louis, MO), as follows. The dried sample was suspended in 2 μL of 1 M sodium cyanoborohydride in tetrahydrafuran (Catalog No. 29,681-3, Sigma-Aldrich) and 2 μL APTS (0.1 mg/μL in 15% acetic acid). The reaction was incubated overnight at 42°C, then diluted with 46 μL water, vortexed, and centrifuged briefly in a microfuge. A 5 μL aliquot, containing 0.4 μg glucose equivalents, was added to 195 μL purified water, and this sample was applied to a Beckman P/ACE capillary electrophoresis instrument. The sample injection parameters were 5 s at 0.5 psi. Separation was accomplished at 23.5 kV in an uncoated capillary using Carbohydrate Separation Gel Buffer N (Catalog Nos. 338451 and 477623, respectively, Beckman Coulter, Inc., Fullerton, CA). Results shown are the average chain length distributions of amylopectin isolated from four separate plants. Differences between samples were statistically insignificant.

The structure of WSP from kernels was determined similarly. The only differences were that some samples of WSP were not treated with any debranching enzyme prior to APTS labeling, whereas others were digested with a combination of the isoamylase

described in the preceding paragraph and 0.05 units of *Klebsiella pneumoniae* pullulanase (Catalog No. P-5420, Sigma-Aldrich).

Germination Analysis

Seeds were surface sterilized by immersion in 1% sodium hypochlorite for 5 min, then washed three times with sterile water. Fifteen seeds from each of three ears were placed on Petri dishes containing three layers of moist Whatman paper and incubated at 30°C in the dark. The lengths of the 15 cotyledons of each genotype were measured on successive days throughout the incubation period. To measure the amount of starch, the roots, cotyledons, and pericarps were removed from three kernels, and the endosperms were then dissected and ground in a mortar and pestle. The ground tissue was washed in water, centrifuged at 1,500*g* for 10 min and then suspended in 4 mL water. For normalization of samples, 1 mL of this suspension was dried and weighed. The remaining 3 mL of the suspension was boiled for 30 min, and the total glucan polysaccharide in the soluble phase was quantified as described above for leaf tissue.

Protein Analysis

To isolate proteins from kernels, leaves, and germinating seeds, five grams of tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle, and the tissue was suspended in 5-7 mL of buffer containing 50 mM Tris acetate, pH 7.5, 10 mM EDTA, 20 mM DTT. The homogenate were centrifuged at 100,000*g* for 1 h at 4°C. The supernatant was stored at -80°C. Starch zymogram analysis was performed as described (Dinges et al., 2001). For the pullulan azure activity gel, the resolving gel contained 7% (w/v) acrylamide (29:1 acrylamide-bisacrylamide) and 375 mM Tris-HCl, pH 8.8. The

stacking gel contained 4% (w/v) acrylamide and 63 mM Tris-HCl, pH 6.8. Electrophoresis was conducted using approximately 50 μg of sample (except where noted) at 4°C, 25 V cm^{-1} for 2 h in a Mini-Protean II cell (Bio-Rad) in an electrode buffer of 25 mM Tris, 192 mM glycine, pH 8.8, and 2 mM DTT. At the end of the run, the gel was electroblotted to a polyacrylamide gel of the same size containing 7% (w/v) acrylamide, 1% (w/v) pullulan azure (Catalog No. P-0588, Sigma-Aldrich), and 375 mM Tris-HCl, pH 8.8. The transfer was performed overnight at 20 V in the electrode buffer at room temperature.

For zymogram analysis of starch synthase activities, protein samples were separated in a native 6% (w/v) polyacrylamide gel containing 0.2% (w/v) rabbit glycogen (Catalog no. G-8876, Sigma-Aldrich) for 3 h at 4°C, and was incubated overnight and stained as described (Cao et al., 1999). Gels were photographed immediately after staining or transfer. SDS-PAGE/Immunoblot analysis was performed as described (Beatty et al., 1999). Crude anti-SBEIIa was diluted 1:5,000. Anion exchange fractionation of total soluble kernel extracts was performed using a MonoQ HR 5/5 column (Catalog No. 17-0546-01, Amersham Biosciences). The proteins were separated using a linear gradient of 0 to 0.4 M NaCl.

Availability of materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in non-commercial research purposes.

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CHAPTER 4. GENETIC ANALYSIS INDICATES MAIZE PULLULANASE- AND ISOAMYLASE-TYPE STARCH DEBRANCHING ENZYMES HAVE PARTIALLY OVERLAPPING FUNCTIONS IN STARCH METABOLISM

A paper published in the *Journal of Applied Glycoscience*[1]

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Abstract

The functions of the two known plant classes of α(1→6) glucan hydrolase were investigated by genetic analysis in maize. A null mutation in the *Zpu1* gene, coding for the pullulanase-type starch debranching enzyme (DBE), was coupled with three different alleles of the *Su1* gene, coding for the isoamylase-type DBE. In two instances the starch biosynthesis abnormality conditioned by the isoamylase-type DBE mutation, namely a decrease in starch content and an increase in phytoglycogen content, was exacerbated by the additional loss of pullulanase-type DBE activity. These data confirm previous findings that pullulanase-type DBE contributes to starch biosynthesis in developing endosperm. An appreciable level of starch was still formed, however, in mutant endosperm bearing null mutations for both classes of DBE, and in this instance the pullulanse-type DBE had no effect on the amount of starch produced. The data suggest that a critical level of isoamylase-type DBE protein or activity may be required in order for pullulanase-type DBE to be able to

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function productively in starch biosynthesis. Furthermore, the data show clearly that a significant degree of starch biosynthesis, approximately 40% of normal, occurs in the absence of any detectable DBE activity in the endosperm of maize kernels.

Introduction

This study investigated the physiological functions of starch debranching enzymes (DBEs), which hydrolyze $\alpha(1\rightarrow6)$ glycoside bonds in glucose polymers. Plant $\alpha(1\rightarrow6)$ glucan hydrolases can be broadly classified as either "isoamylase-type DBEs" or "pullulanase-type DBEs" (also known as limit dextrinases, or R-enzymes), based upon their *in vitro* substrate specificity as well as amino acid similarity to previously characterized prokaryotic enzymes. Isoamylase-type DBEs hydrolyze $\alpha(1\rightarrow6)$ bonds in glycogen and amylopectin, but are not active towards such linkages in pullulan. Pullulanase-type DBEs, in contrast, readily hydrolyze $\alpha(1\rightarrow6)$ bonds in pullulan, but are inactive or only very slightly active towards glycogen or amylopectin (Lee and Whelan, 971; Doehlert and Knutson, 1991; Wu et al., 2002). Genomic sequences reveal three distinct genes that code for isoamylase-type DBEs in rice and Arabidopsis, and three distinct cDNAs coding for such enzymes are known in maize and potato (Tabata et al., 2000; Yu et al., 2002; Dinges et al., 2003). A single gene coding for pullulanase-type DBE has been identified in the two available genomic sequences, and a single pullulanase-type DBE cDNA has been isolated from maize and other plants (Renz et al., 1998; Beatty et al., 1999; Kristensen et al., 1999; Tabata et al., 2000; Goff et al., 2002; Yu et al., 2002). Phylogenetic analysis shows that isoamylase- and pullulanase-type DBEs have been conserved separately throughout the evolution of plants and bacteria (Beatty et al., 1999). These data support the hypothesis that

the two different types of DBE, despite having the same enzymatic activity, have specific physiological functions that cannot be completely substituted by a member of the other class. The specific function(s) of each DBE class, however, remains to be discerned.

The hydrolytic activity of the DBEs points to an obvious role in starch degradation, but extensive genetic and biochemical analysis in many plant species has shown that mutations in these enzymes affect starch anabolism as well as its breakdown. Mutations in one of the isoamylase-type DBE genes, coded for by the *Sugary1* (*Su1*) gene of maize (James et al., 1995) and rice (Fujita et al., 1999) and homologs in Arabidopsis (Zeeman et al., 1998), barley (Burton et al., 2002), and *Chlamydomonas* (Mouille et al., 1996), result in decreased starch amounts and accumulation of a highly-branched, water soluble polysaccharide (WSP) termed phytoglycogen (PG). Three models have been proposed to explain the accumulation of PG in DBE mutants and, accordingly, the function of these enzymes in starch synthesis. The glucan trimming model proposes that DBEs are directly involved in the determination of the amylopectin (Ap) fine structure by removing extraneous branches from a nascent Ap chain, thereby facilitating crystallization (Ball et al., 1996; Myers et al., 2000). In the absence of such activity, branching may reach a level where crystallization is no longer possible, leading to PG accumulation. A second model suggests that DBEs may be involved in starch synthesis indirectly by clearing soluble glucan, i.e. polymer accumulating separately from the granule, that would otherwise divert enzyme and substrate from the productive synthesis occurring at the granule surface (Zeeman et al., 1998; Smith, 2001). A third model recently was proposed that suggests the DBEs are necessary for proper starch granule initiation (Burton et al., 2002). Uncontrolled granule initiation could lead to multiple, compound granules and phytoglycogen accumulation.

The pullulanase- and isoamylase-type DBEs may be functionally related. Studies of *su1*⁻ mutants of maize and rice revealed that loss of isoamylase-type DBE causes a concomitant reduction in the activity of the pullulanase-type DBE as a secondary, pleiotropic effect (Pan and Nelson, 1984; Beatty et al., 1999; Wu et al., 2002). By correlating starch-containing regions of *su1*⁻ mutant rice endosperm with residual pullulanase-type DBE activity, Nakamura et al. speculated that the pullulanase-type DBE, not the isoamylase-type DBE, is the primary determinant of the phytoglycogen accumulating phenotype (Nakamura et al., 1997; Kubo et al., 1999). Pleiotropic effects on pullulanase-type DBE activity, however, do not result from isoamylase mutations in Arabidopsis, barley, or *Chlamydomonas* (Mouille et al., 1996; Zeeman et al., 1998; Burton et al., 2002). Wu et al. have speculated that the reduction of pullulanase-type DBE activity in isoamylase-type DBE mutants is caused by post-translational modification of the enzyme, perhaps resulting from increased cellular sucrose concentrations (Wu et al., 2002).

The functional relationships between the two types of DBE were addressed recently by use of a transposon insertion mutation in the gene *Zpu1* (Dinges et al., 2003). The mutant allele *zpu1-204* caused complete loss of pullulanase-type DBE activity, and resulted in defects in starch degradation in leaves as well as germinating seeds. The mutation alters the metabolism of maltooligosaccharides (MOS) in the developing kernel, but does not lead to production of detectable quantities of PG. In itself, this fact suggests that the pullulanase-type DBE is not the primary determinant of the PG-accumulating phenotype in maize endosperm. However, when combined with a specific allele of *su1, su1-starchy* (*su1-st*), the *zpu1* null mutation causes a five-fold increase in the amount of PG. Thus there appears to be some functional overlap between the two DBE isoforms (Dinges et al., 2003).

In this study, genetic methods were employed to eliminate pullulanase-type DBE activity in a series of maize lines containing various mutations of the *Su1* gene, each with distinct effects on the isoamylase-type DBE and the PG accumulation phenotype. The data confirmed that pullulanase-type DBE can affect starch biosynthesis, because in the presence of several *su1* alleles that reduce but do not completely eliminate isoamylase-type DBE activity, the pullulanase-type DBE determined the amount of PG versus granular starch produced. Of further interest was to test the effects of combining null mutations of both *zpu1* and *su1* in single plants, thus eliminating all detectable DBE proteins and activity in developing endosperm. This experiment revealed that the ability of pullulanase-type DBE to influence starch versus PG content requires the presence of a critical threshold level of isoamylase-type DBE activity or protein, indicative of a specific function for the latter isoform in starch biosynthesis. Furthermore, granular starch accumulated in the double mutants to the same extent observed in the null *su1*$^{-}$ single mutant, thus ruling out the possibility that residual granule formation in isoamylase-type DBE mutants results from partial substitution of an isoamylase-type DBE function by the pullulanase-type DBE activity.

Results and Discussion

Generation of double mutant kernels

To analyze the functional interaction between the maize pullulanase- and isoamylase-type DBEs, seeds carrying mutations affecting both enzyme isoforms were generated (Figure 4.1). Singly homozygous maize plants bearing the pullulanase-type DBE mutation *zpu1-204* were crossed to plants homozygous for the isoamylase-type DBE mutant alleles *su1-5051,*

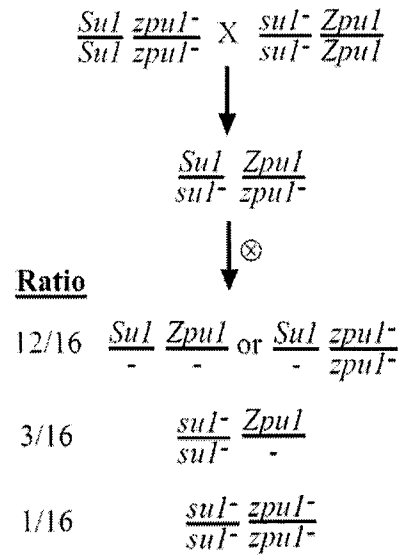

Figure 4.1. Crossing scheme used to generate *su1*⁻ *zpu1*⁻ double mutants. Singly homozygous plants were crossed and the resulting compound heterozygote was self-pollinated to give the F_2 ear. These ears segregate for the doubly homozygous mutant kernels in a ratio of approximately one-sixteenth. Dashes (-) indicate that either the nonmutant allele or the mutant allele can be present in kernels of the particular phenotypic class.

su1-Bn2, or *su1-4582*. The progeny plants from this cross were heterozygous at both the *zpu1* and *su1* loci, so that their self-pollination generated ears on which kernels segregated independently for both mutations. One-fourth of the kernels are expected to be homozygous for the recessive *su1*⁻ mutant allele and thus to display a discernable sugary phenotype, i.e., shrunken kernels with a glassy appearance. One-fourth of the kernels also are expected to be homozygous for *zpu1-204*, however, this defect does not condition a noticeable kernel phenotype (Dinges et al., 2003). As expected, in all instances three-fourths of the kernels on the ear displayed a wild type appearance, and one-fourth displayed the sugary phenotype to varying degrees (Figure 4.2).

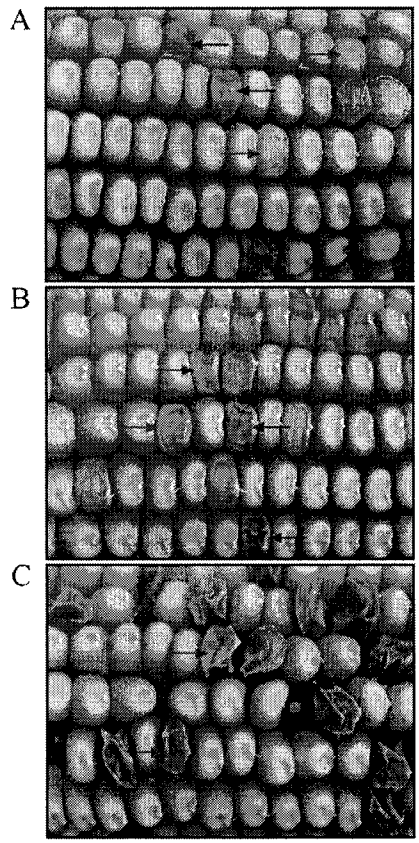

Figure 4.2 Kernel phenotypes of *su1⁻ zpu1⁻* double mutants. **A.** A portion of the ear resulting from the self pollination of a *Su1/su1-5051; Zpu1/zpu1-204* compound heterozygote is shown. Approximately three-sixteenths of the kernels possess the moderate kernel phenotype typical of the su1-5051 homozygous parent (green arrows). A more severe sugary kernel phenotype appears in approximately one-sixteenth of the progeny (red arrows). **B.** A portion of the ear resulting from the self-pollination of a *Su1/su1-Bn2; Zpu1/zpu1-204* compound heterozygote. The moderate kernel phenotype typical of the *su1-Bn2* parent appears in approximately three-sixteenths of the progeny (green arrows) and a more severe phenotype occurs in about one-sixteenth of the progeny (red arrows). **C.** A portion of the ear resulting from the self-pollination of a *Su1/su1-4582; Zpu1/zpu1-204* compound heterozygote. Approximately one-fourth of the kernels possess the extremely shrunken and glassy kernel phenotype of the *su1-4582* parent (green arrows). No obvious difference can be detected between the kernels that are also homozygous for *zpu1-204* and those that are not.

The allele *su1-5051* conditions a moderate phenotype in which kernels are slightly collapsed at the crown, and this was observed in approximately three-sixteenths of the kernels on the ear (Figure 4.2A green arrows). One-sixteenth of the kernels displayed a

more severe shrunken and glassy phenotype not typical of *su1-5051* mutants (Figure 4.2A, red arrows), and these were presumed to be *su1-5051*, *zpu1-204* double homozygotes. The genotype at the *zpu1* locus was determined by PCR amplification of genomic DNA (Dinges et al., 2003) from embryos of five individual kernels with the moderate phenotype and five others with the more severe phenotype. In all instances the severe kernels were confirmed to be homozygous for *zpu1-204*, whereas all of the less severely affected kernels contained at least one wild type *Zpu1* allele (data not shown). The endosperm tissue from the same kernels that were used for genotype determination was saved for determination of granular and soluble glucan content. Thus this population of kernels allowed comparison of congenic endosperm tissue that differed only by the presence or absence of a functional *Zpu1* allele.

Similar results were obtained for the kernel population segregating for *su1-Bn2* and *zpu1-204*, again with three-sixteenths of the progeny displaying a relatively weak sugary phenotype (Figure 4.2B, green arrows) and one-sixteenth with an enhanced phenotype (Figure 4.2B, red arrows). In this cross the enhanced phenotype was characterized by a more collapsed kernel crown and by a slightly darker color than the typical moderate phenotype conditioned by *su1-Bn2*. Genotype determination by PCR again confirmed that the more severely affected kernels were homozygous for *zpu1-204* and that the less severely affected kernels all contained at least one wild type *Zpu1* allele (data not shown). From the cross involving the null allele *su1-4582* there was no visual distinction possible among the sugary kernels, all of which were severely affected to the same extent (Figure 2C). Homozygous *zpu1-204* kernels were distinguished by PCR analysis from sibling seeds that contained at least one copy of the functional *Zpu1* allele (data not shown).

Soluble and granular carbohydrate distribution in single kernels

As described above, the genotypes of mature, dried kernels were determined from analysis of the dissected embryos, and endosperm from the same individual kernels was used to determine the percentage of glucan polymers in the soluble and granular phases. In the cross involving *su1-5051*, the segregant kernels with wild type appearance (presumed to contain at least one wild type *Su1* allele) contained less than 1% of their total α-glucan polymer in the form of WSP (Table 4.1).

Table 4.1. Carbohydrate content of individual DBE mutant kernels[a]

Kernel Genotype	No. of Kernels Analyzed	% Starch	% WSP	± SD
Segregants of *su1-5051* x *zpu1-204*:				
Su1/-; Zpu1/- or *Su1/-; zpu1⁻/zpu1⁻*	5	99.4	0.6	0.3
su1⁻/su1⁻; Zpu1/-	5	86.6	13.4	2.0
su1⁻/su1⁻; zpu1⁻/zpu1⁻	5	50.3	49.7	5.8
Segregants of *su1-Bn2* x *zpu1-204*:				
Su1/-; Zpu1/- or Su1/-; zpu1⁻/zpu1⁻	5	98.8	1.2	0.3
su1⁻/su1⁻; Zpu1/-	7	74.9	25.1	6.4
su1⁻/su1⁻; zpu1⁻/zpu1⁻	3	53.1	46.9	1.3
Segregants of *su1-4582* x *zpu1-204*:				
su1⁻/su1⁻; Zpu1/-	15	37.8	62.2	8.3
su1⁻/su1⁻; zpu1⁻/zpu1⁻	5	36.3	63.7	6.0

[a] Values for percent starch and percent WSP are normalized to the total glucan polymer content in each individual kernel that was analyzed.

In previous analyses the WSP from wild type kernels was shown to be small maltooligosaccharides as opposed to PG (Dinges et al., 2001). The *su1-5051* single mutant kernels (*su1-5051/su1-5051; Zpu1/Zpu1* or *su1-5051/su1-5051; Zpu1/zpu1-204*) possessed higher levels of WSP than the wild type kernels, amounting to 13.4% of the total α-glucan polymer, and accordingly exhibited a statistically significant reduction in the amount of starch present (Table 4.1). The double mutant kernels (*su1-5051/su1-5051; zpu1-204/zpu1-204*) possessed markedly more WSP than the *su1-5051* single mutants, approximately 50% of the total α-glucan polymer (Table 4.1). These data suggest that in the *su1-5051* mutant, the pullulanase-type DBE prevents accumulation of large amounts of PG and thereby partially substitutes for the reduction in isoamylase-type DBE. An increase in PG content of about this magnitude was also observed previously in the *su1-st; zpu1-204* double mutant compared to the *su1-st* single mutant (Dinges et al., 2003).

Similar results were obtained from the crosses between *zpu1-204* plants and those bearing a slightly more severe *su1*$^{-}$ allele, *su1-Bn2*. Wild type kernels from this cross were again found to contain very low levels of WSP, approximately 1% of the total α-glucan polymer (Table 4.1). The single mutant kernels (*su1-Bn2/su1-Bn2; Zpu1/Zpu1* or *su1-Bn2/su1-Bn2; Zpu1/zpu1-204*) were found to contain 25.1% WSP, whereas the double mutant kernels (*su1-Bn2/su1-Bn2 zpu1-204/zpu1-204*) contain approximately twice that amount. Although the two-fold increase is modest compared to the results of coupling *zpu1-204* with *su1-5051* (Table 4.1) or *su1-st* (Dinges et al., 2003), the difference clearly is statistically significant (p-value < 0.05).

To specifically examine the effect on starch metabolism of complete loss of both isoamylase- and pullulanase-type DBE, crosses were made to generate double null mutant

seeds. The *su1-4582* mutation contains a *Mu1* transposable element in the first exon of the gene (James et al., 1995). As expected, mRNA transcript is undetectable in seeds homozygous for this allele (Dinges et al., 2001), nor is any isoamylase-type DBE activity observed in developing mutant endosperm (J. Dinges and C. Colleoni, unpublished results). On segregating ears, there was no apparent phenotypic difference between kernels homozygous for only *su1-4582* and those homozygous for mutations at both the *su1* and *zpu1* loci (Figure 4.2C). Single-kernel carbohydrate analysis and genotyping of 20 sugary kernels from a segregating ear revealed those seeds which were, in fact, double mutants (Table 4.1). The single mutant *su1-4582* kernels contained 62.2% WSP and the double mutant *su1-4582; zpu1-204* kernels contained 63.7% WSP (Table 4.1), a difference that is statistically insignificant (p-value > 0.05). Thus, in the case of the *su1-4582* mutation, the presence or absence of pullulanase-type DBE activity does not affect the level of PG versus granular starch produced.

Interaction between isoamylase- and pullulanase-type DBEs

This study made use of three allelic mutations in the *su1* locus, which differ in the severity of the phenotype that results as it is measured both by the visible morphology and the ratio of WSP to granular starch. The most severe mutation is the null allele *su1-4582*, which completely eliminates the mRNA, protein, and enzymatic activity that correspond to this particular isoamylase-type DBE. The molecular defect in *su1-5051* is caused by a *Mu1* transposable element inserted into the 5' untranslated region, at nucleotide position 1964 of the published genomic sequence (Genbank accession no. AF030882) (J. Dinges, unpublished data). Genes with such a structure are termed *Mu*-suppressible (Barkan and Martienssen,

1991), because transcription is not entirely eliminated. Rather, a promoter within the *Mu* element allows for transcription at a level that is reduced compared to wild-type (data not shown). Thus *su1-5051* is likely to result in diminished production of the SU1 protein relative to wild type, but that protein should have the normal amino acid sequence. The molecular defect of *su1-Bn2* is a single nucleotide change resulting in substitution of the asparagine at position 627 by a lysine residue (J. Dinges, unpublished results). In this instance, therefore, normal gene expression is expected, although the product will have an altered primary sequence.

Elimination of pullulanase-type DBE activity had an effect on starch content in the presence of the two weaker *su1*$^-$ alleles, *su1-5051* and *su1-Bn2*, but not in *su1-4582* kernels completely lacking *su1* function. Based on these results, we conclude that some degree of isoamylase-type DBE function is required in order for the pullulanase-type DBE to be able to affect starch biosynthesis. Above a predicted threshold level the glucan biosynthesis system might proceed to the point where further $\alpha(1\rightarrow6)$ glucan hydrolase activity from the pullulanase-type DBE could cause a higher degree of partitioning of polymer into the granular phase, whereas below this level either pullulanase-type DBE activity is not possible, or is not productive in terms of granule formation. A possible explanation of this finding is that there is a critical level of isoamylase-type DBE activity that is needed in order to form a substrate on which the pullulanase-type DBE could act. Alternatively, the isoamylase-type DBE protein might have a direct or indirect effect on the enzymatic properties of the pullulanase-type DBE. The alleles that produce either reduced levels of the *su1* gene product, or a protein with an altered primary sequence, would be able to provide the requisite isoamylase-type DBE function, whereas the null allele would not.

The data reported here confirm the previous conclusion that the isoamylase-type DBE and the pullulanase-type DBE are capable of working synergistically in normal kernels to prevent larger amounts of PG from accumulating. It is not obvious, therefore, why the complete or nearly complete loss of DBE activity in the *su1-4582; zpu1-204* double homozygotes does not result in the complete replacement of granular starch by PG. Three reasons might be offered to explain starch formation in the double mutant kernels. First, starch synthesis may be a completely separate process from PG accumulation, as suggested in the WSP clearing model proposed by Zeeman and colleagues (Zeeman et al., 1998). DBE activity may not be needed for the formation of granular starch, but instead it may be required for preventing the diversion of substrate and enzyme to synthesis in the soluble phase. The approximately 40% residual starch present in the double mutant may be the equilibrium point in the competition for glucan synthesis in the soluble and granular phases. A second explanation for the residual starch is that some degree of isoamylase-type DBE function is being provided by one or both of the two additional genes present in the maize genome that code for members of this enzyme class. Although their activities have not been observed on native PAGE/activity gels, the possibility exists that these enzymes are contributing to starch metabolism in the maize endosperm. Such activity might be sufficient to allow for low level granular starch formation, but not provide whatever minimal function is needed to enable the pullulanase-type DBE to contribute further to the formation of granular starch. Finally, the remaining starch in the *su1-4582; zpu1-204* endosperm may be created due to random crystallization events that occur at the surface of the starch granule. Myers et al. speculated that crystallization would be an entropically-driven process, which

would be facilitated by removal of extraneous branches by DBEs (Myers et al., 2000). The model predicts that some crystallization could occur in the absence of DBE activity.

Although these data show that both DBE isoform classes influence the degree of phytoglycogen accumulation, they do not distinguish the exact nature of the overall function of DBEs in starch synthesis, nor do they imply that both DBE isoforms necessarily must have the same function. An overlapping function in a single step in starch biosynthesis is consistent with the data. Separate functions, however, also are consistent with the observations. For example, a role for the isoamylase-type DBE in glucan trimming does not preclude the pullulanase-type DBE from acting in WSP clearing, or visa versa. Thus the possibility exists that the two DBE isoforms are involved in two distinct processes, both of which contribute to the partitioning of glucans between the soluble- and the granular phases.

Materials and Methods

Plant material

All maize plants (*Zea mays* L.) used in this study were in the standard inbred W64A genetic background. The null mutations *zpu1-204* and *su1-R4582::Mu1* (*su1-4582*) were described previously (James et al., 1995; Dinges et al., 2001; Dinges et al., 2003). Seeds containing the allele *su1-R5051::Mu1* (*su1-5051*) were provided by Dr. Donald S. Robertson (Iowa State University, Ames, IA), and *su1-Brawn2* (*su1-Bn2*) (Brink, 1984) seeds were obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL). All plants used in the study were grown under field conditions in Ames, IA.

Carbohydrate extraction and quantification

Mature, dried kernels were imbibed in 0.3% (w/v) sodium metabisulfite, 1% (v/v) lactic acid overnight at 50°C. The pericarp and embryo were removed by manual dissection. Endosperm tissue from individual kernels was homogenized in a microfuge tube using a pestle and suspended in 0.5 mL H_2O. The homogenate was centrifuged at 3,000*g* for 5 min at room temperature, and the pelleted material was resuspended with 0.5 mL H_2O and centrifuged two times additional times. The supernatants from each centrifugation were pooled together. Endosperm starch and WSP in the pellet and supernatant fractions, respectively, were measured as described previously (Dinges et al., 2001). Results are expressed as percent starch or WSP of total α-glucan polymer per endosperm.

Kernel genotype determination

DNA was isolated from embryos of individual kernels following standard procedures (Saghai-Maroof et al., 1984). The genotype was determined using polymerase chain reaction (PCR) amplification with primers specific for either the wild-type *Zpu1* allele or the mutant *zpu1-204* allele as described (Dinges et al., 2003).

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CHAPTER 5. GENE EXPRESSION ANALYSIS OF MAIZE STARCH DEBRANCHING ENZYMES BY REAL-TIME QUANTITATIVE PCR.

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Abstract

This study describes the full complement of maize starch debranching enzymes (DBE) or α(1→6) glucan hydrolase, and their expression patterns. Plants contain two conserved types of DBE, the isoamylase- and pullulanase-type enzymes. Genetic analysis of *sugary1* (*su1*) mutations in maize and other cereals indicates that at least one isoamylase-type DBE is required for normal starch biosynthesis. Genetic analysis of maize *Zpu1*, coding for the pullulanase-type DBE, suggests functions both in starch degradation and starch biosynthesis, and that the biosynthetic function overlaps partially with that of the isoamylase-type DBE coded for by *Su1*. Here we report the cloning of two additional isoamylase-type DBE cDNAs, *ZmIso2* and *ZmIso3*. Like *Su1* and *Zpu1*, these sequences have been conserved throughout the evolution of plants. A comprehensive quantitative analysis of the expression of all four DBE transcripts in metabolically active tissues showed that DBEs are expressed ubiquitously throughout the plant, although to varying levels. *Su1* is the most highly expressed mRNA, and is present during times of net starch synthesis. The pattern of *Su1* expression follows closely that of *ZmIso2*, consistent with the idea that these

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isoforms form a heteromultimer. The expression of *ZmIso3* is most abundant in germinating seed, consistent with a role for this isoform in starch degradation. Thus, individual DBE isoforms appear to be dedicated to specific catabolic and/or anabolic functions.

Introduction

Starch serves a fundamental role in the life cycle of the plant as the primary carbohydrate storage form. Starch is synthesized when energy is abundant and degraded when energy is required. Two forms of starch, storage and transient, are classified based upon the length of time they are stored. Storage starch accumulates in heterotrophic tissues and serves the long-term needs of the plant during germination or sprouting. In contrast, transient starch accumulates and degrades over a short period of time, usually a single day, as in the diurnal cycle of leaf starch metabolism.

Starch is a mixture of two polymers of α-D-Glucose, amylose and amylopectin, Amylose contains few, if any branches, whereas amylopectin is more highly branched, containing approximately 5% α(1→6) linkages. The synthesis of starch is dependent upon numerous isoforms of starch synthase (SS), branching enzyme (SBE), and debranching enzyme (DBE) (Myers et al., 2000; Smith, 2001). The relative contribution of each enzyme isoform to the process of starch synthesis is not known, and the mechanism by which these enzymes create the complex architecture of the starch granule remains to be discerned.

Plants contain two types of DBE, or α(1→6) glucan hydrolase, which are classified based upon their substrate specificity and amino acid similarity to defined bacterial proteins. Isoamylase-type DBEs cleave linkages in glycogen and amylopectin, but not the repeating isomaltotriose polymer pullulan. Pullulanase-type DBEs (also known as limit dextrinases)

cleave linkages in pullulan and limit dextrins, but are not active or only slightly active toward amylopectin and glycogen (Manners, 1971).

Biochemical and genetic studies in several species have shown that DBEs perform a dual function in starch metabolism. The hydrolytic activity of DBEs points toward an obvious role in starch catabolism, as these are the starch degradative enzymes that efficiently cleave $\alpha(1\rightarrow6)$ linkages in glucose polymers. However, DBEs are also expressed to a significant extent in tissues and developmental stages where there is net starch synthesis. Rather than simply being stored for use during degradation, mutant studies have shown that DBEs perform a necessary function during the synthesis of starch. For example, loss of isoamylase-type DBE in maize, Chlamydomonas, rice, Arabidopsis, and barley result in the accumulation of phytoglycogen, a soluble glucan polymer, instead of the normal crystalline starch (James et al., 1995; Mouille et al., 1996; Nakamura et al., 1996; Zeeman et al., 1998; Burton et al., 2002). Mutational analysis of the pullulanase DBE of maize also indicates a dual function in starch anabolism. Plants lacking pullulanase-type DBE also accumulate branched, soluble maltooligosaccharides (MOS) during kernel starch formation, which is not found in the wild-type. The phenotypic severity of mutant alleles of *Su1*, a gene coding for isoamylase, is also increased when pullulanase-type DBE is lacking, suggesting an overlapping function for the two types of DBE (Dinges et al., 2003a, Dinges et al., 2003b).

The hydrolytic activity of DBEs suggests a role in starch catabolism. However, the alteration in carbohydrate composition during kernel development, owing to a defective DBE, does not suggest an obvious function for these enzymes in the process of starch anabolism. To explain the mutant phenotype, the glucan trimming model proposes that DBEs are required to remove extraneous branches from a pre-amylopectin molecule, thereby

facilitating crystallization into starch (Ball et al., 1996; Myers et al., 2000). As a consequence of DBE deficiency, pre-amylopectin fails to crystallize appropriately, which leads to accumulation of phytoglycogen. A second hypothesis, the water soluble polysaccharide (WSP) clearing model, proposes that DBEs act separate from the synthesis of the starch granule to degrade soluble glucan, preventing it from accumulating in the stroma (Zeeman et al., 1998). Currently available evidence does not irrefutably support one model versus the other. Nevertheless, both models agree that a function provided by DBEs is required for the proper synthesis of starch.

The complete genome sequences of Arabidopsis and rice contain three isoamylase-type DBE sequences and one pullulanase-type DBE sequence (Yu et al., 2002). Members of the isoamylase and pullulanase-type DBE classes have been maintained separately throughout evolution, suggesting specific functions for each of these isoforms, leading to their independent conservation (Beatty et al., 1999). However, the precise contribution(s) of each isoform to starch degradation and/or synthesis remains to be determined. A specialized spatial or temporal expression pattern is also a possible explanation for the high degree of conservation.

This study identified and cloned additional isoamylase-type DBE cDNAs from maize, designated *ZmIso2* and *ZmIso3*. Sequence comparisons reveal that isoamylase-type DBEs fall into three distinct clades, all of which have been separately conserved throughout the evolution of higher plants. Furthermore, a single pullulanase-type DBE is present in the species of the plant kingdom examined to date. To examine potential sub-functionalization of the DBE isoforms, expression of the genes was investigated using real-time quantitative polymerase chain reaction (PCR). All isoforms were found to be expressed in tissues that

were active in starch metabolism, i.e. during kernel starch formation and the period of starch degradation during germination. Other tissues, such as leaf, root, and tassel also show expression of DBEs to varying levels, indicating that this gene family is expressed nearly ubiquitously, underlying the essential metabolic and developmental functions that starch serves for the plant.

Results and Discussion

Identification and Cloning of Maize Isoamylase cDNAs

Previous genetic and biochemical studies of developing maize endosperm have revealed that the major form of isoamylase-type DBE is coded for by the *Su1* gene (James et al., 1995; Rahman et al., 1998). Null mutations of *Su1* cause the complete elimination of detectable isoamylase activity in protein extracts from developing endosperm (Dinges et al., 2001). However, two isoamylase activity peaks were observed following anion exchange chromatography of wild-type extracts, providing some empirical evidence that multiple forms of isoamylase may exist (Doehlert and Knutson, 1991). The fact that the additional forms of isoamylase do not contribute to any residual activity in *su1*- mutants suggests that they lack detectable activity or be dependent on the SU1 isoform for function.

Other evidence for the existence of additional isoamylase isoforms comes from the complete genome sequences of Arabidopsis and rice (*Oryza sativa*). BLAST searches revealed three putative genes coding for isoamylase-like proteins in rice. One sequence was identical to that previously reported as the rice *Sugary1* gene (*OsIso1,* Fujita et al., 1999). Two other genes encoded predicted proteins having 50% and 56% amino acid similarity to OsISO1, and were designated *OsIso2* and *OsIso3*, respectively. The rice isoamylase genes

OsIso2 and *OsIso3* possess markedly different gene structures. *OsIso2* is intronless, with the coding region contained within a single exon. In contrast, *OsIso3* possesses 20 introns, spanning >10 kb of genomic sequence. These gene structures appear to be conserved since similar intron/exon boundaries are delineated in the Arabidopsis homologs *AtIso2* and *AtIso3* (data not shown). Orthologous sequences have also been reported in potato, named *StIsa2* and *StIsa3* (Hussain et al., 2003).

Most gene discovery approaches have been largely unsuccessful in identifying additional DBE sequences in maize. However, searches of the Pioneer Hi-Bred maize EST collection identified one partial cDNA clone corresponding to an isoamylase-like sequence that was similar to *OsIso3*, and thus designated *ZmIso3*. Since maize and rice are both members of the family *Poaceae*, there was a strong possibility that DNA sequence similarity could be used to clone the maize orthologs of *OsIso2* and *OsIso3*. Third-codon degenerate oligonucleotide primers were designed from the *OsIso2* and *OsIso3* sequences. This is based on the prediction that conservation of these sequences would permit amplification of the cDNAs from maize RNA samples. Primer pair JD37/JD42 was designed to amplify the central portion of the *OsIso2* gene and primer pair JD21/JD24 was designed to amplify the central portion of the *OsIso3* cDNA. These primers were used in reverse transcriptase (RT)-PCR amplifications of RNA from developing maize kernels. Both primer pairs successfully amplified regions of the maize orthologs (data not shown), and sequencing of the PCR products identified the cDNAs as *ZmIso2* and *ZmIso3*. 5′ and 3′ rapid amplification of cDNA ends (RACE) was used to obtain full-length sequence information for both *ZmIso2* and *ZmIso3* (Figure 5.1). ZmISO2 and ZmISO3 are 83% and 90% similar at the amino acid level to their orthologous sequences in rice.

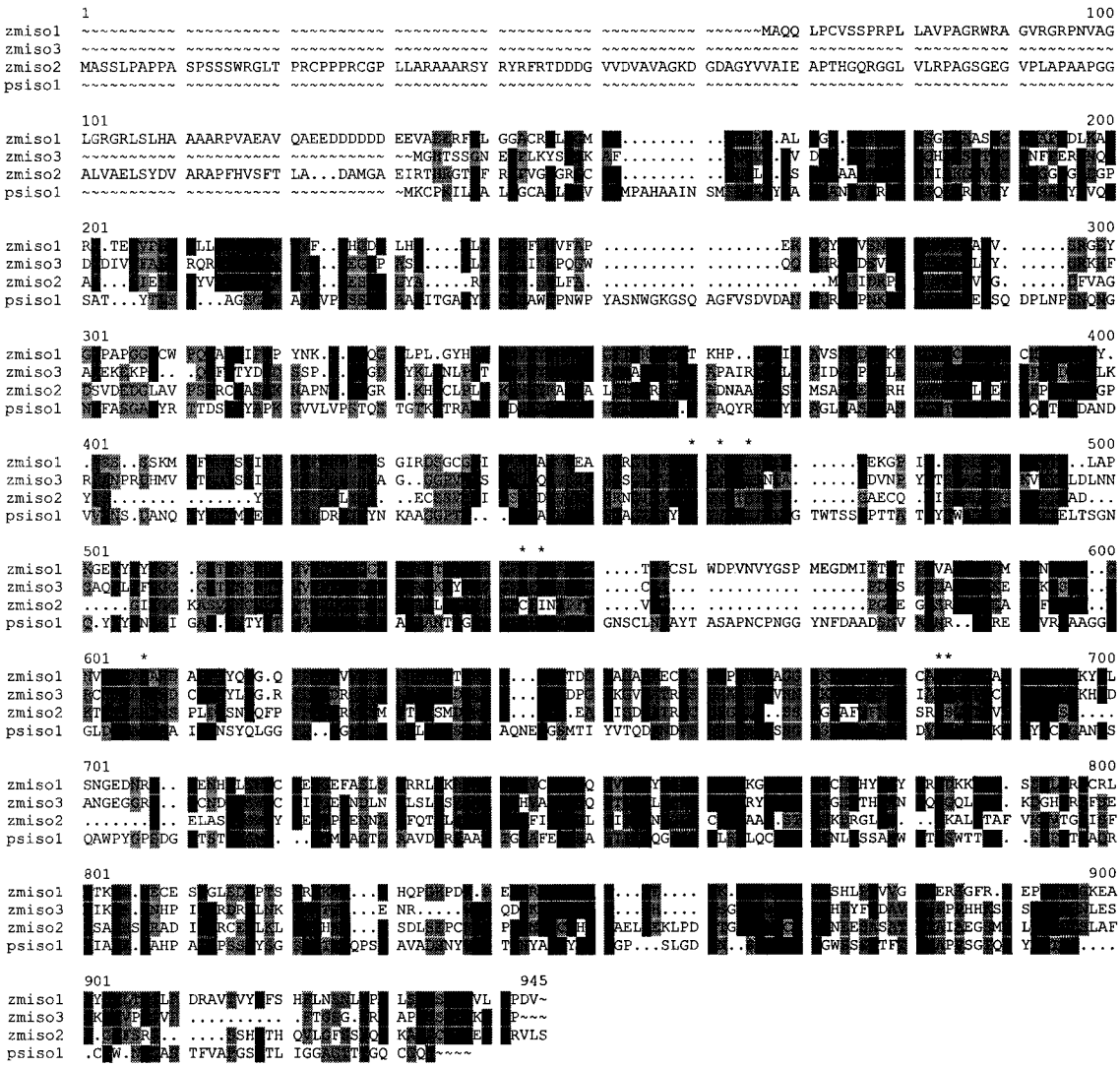

Figure 5.1. Sequence alignment of maize isoamylase-type DBE proteins. Maize isoamylase-type DBEs and *Pseudomonas amyloderamosa* isoamylase were aligned using Pileup. The eight residues predicted to be essential for catalytic activity are indicated by asterisks.

In contrast to the multiple isoamylase-type DBE genes, BLAST searches of the complete genome sequences of rice and Arabidopsis and the EST databases available for other plants revealed a single pullulanase-type DBE isoform in each species, which is coded for by the *Zpu1* gene in maize (Beatty et al., 1999).

Phylogenetic Analysis of Plant and Bacterial DBE Genes

Phylogenetic analysis of selected plant and bacterial DBEs was performed to investigate the evolutionary conservation and relatedness of DBE isoforms (Figure 5.2). The two types of DBE, isoamylase- and pullulanase-type, represent distinct classes of glucan hydrolase. Independent conservation for each type of DBE is suggested by comparisons between the two classes within a single species. For example, the maize isoamylase SU1 has less than 20% amino acid identity to the maize pullulanase ZPU1, but is 45% identical to *Pseudomonas amyloderamosa* isoamylase. Thus, isoamylases and pullulanases represent discrete groups of DBEs that have been conserved separately throughout the evolution of bacteria and higher plants.

Plant isoamylase forms are separated into three major clades, namely the Iso1, Iso2, and Iso3 clades, whereas the bacterial isoamylases represent a separate, but closely related clade (Figure 5.2). These data suggest that two gene duplication events occurred early in the evolution of plants giving rise to three forms of isoamylase. *Synechocystis sp.* possesses two isoamylase-like sequences, *SyIso1* and *SyIso2*. *SyIso1* is more closely related to the bacterial isoamylases, whereas *SyIso2* is more similar to the plant Iso1-type DBEs. Thus, this cyanobacterium possesses both plant- and bacteria-like DBEs, yet accumulates glycogen instead of starch.

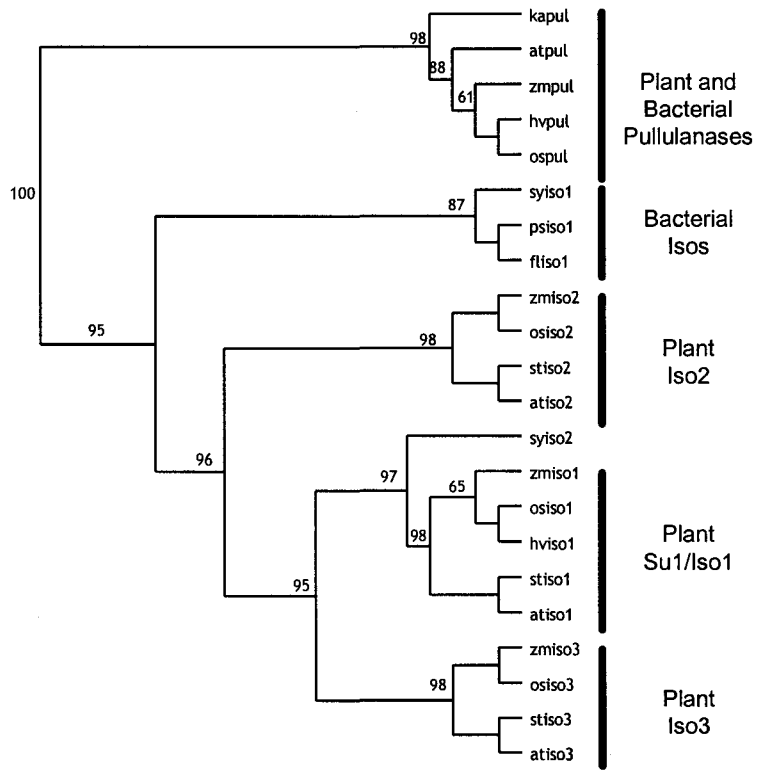

Figure 5.2. Phylogenetic relationships of plant and bacterial DBEs. The parsimony tree was inferred from aligned amino acid sequences using PAUP. Bootstrap values of 100 replicates are indicated. Abbreviations: *Arabidopsis thaliana* (at), *Flavobacterium sp.* (fl), *Hordeum vulgare* (hv), *Klebsiella aerogenes* (ka), *Oryza sativa* (os), *Pseudomonas amyloderamosa* (ps), *Solanum tuberosum* (st), *Synechocystis sp.* (sy), *Zea mays* (zm).

Isoamylases possess 14 conserved domains, four of which are common to all members of the α-amylase superfamily of starch hydrolytic enzymes, (Jespersen et al., 1991; Beatty et al., 1999). Within these conserved regions, eight amino acid residues have been identified as critical for catalytic activity, as they comprise the active site of the protein: Asp-292, Val-294, His-297, Arg-343, Asp-375, Glu-435, His-509, and Asp-510 (Jespersen et al., 1993, based on *Pseudomonas* isoamylase numbering). Most isoamylases exhibit a high degree of conservation of these 8 residues (Figure 5.1); however, the Iso2 form of maize,

rice, potato, and Arabidopsis, shows variability at 6 of 8 of these critical residues. Some of these changes represent conservative substitutions (e.g. D292E and E435D), but others would likely interfere with enzyme catalysis (e.g. D375I, R373C, H509N and D510S). The fact that the Iso2 forms in all species exhibit variability at theses sites suggests that these substitutions occurred before the divergence of monocots and dicots. Consistent with the hypothesis proposed for potato isoamylase (Hussain et al., 2003), *ZmIso2* may perform a function independent of catalytic activity, such as regulation of the *Su1* isoamylase or interaction with other starch biosynthetic proteins in a multimeric enzyme complex.

Expression of DBEs in Developing Kernels

A function in endosperm starch biosynthesis is suggested for some DBE isoforms, since mutations of the isoamylase-type DBE *Su1* result in profound changes in kernel carbohydrate composition (Dinges et al., 2001) and mutations of the pullulanase-type DBE *Zpu1* can also exert effects on carbohydrate composition in some backgrounds (Dinges et al., 2003). To investigate the timing and quantity of DBE transcripts throughout kernel development, real-time quantitative PCR was performed on samples of whole kernels collected on successive days after pollination (DAP). Real-time PCR is a method that relates starting template quantity to a statistically significant increase in fluorescence, i.e. a greater number of transcripts results in the fluorescence reaching the threshold at an earlier cycle during the PCR reaction. Oligonucleotide primers specific to each DBE cDNA were used in the relative quantitation experiments. Verification of primer efficiency and statistical support for the data are described in the Materials and Methods section.

Previous reports examining the expression of starch biosynthetic and storage product genes during kernel development shows a peak mRNA level for many transcripts at approximately 20-24 DAP (Gao et al., 1996; Giroux et al., 1994; Prioul et al., 1994). Like these genes, steady-state levels of DBE mRNA also reach peak levels at 20 DAP, as measured by real-time PCR (Figure 5.3A). *Su1* is the most highly expressed DBE, but the pattern of expression is closely matched by *ZmIso2* and *Zpu1,* although at a reduced amount. The *ZmIso3* cDNA is expressed at an extremely low level in developing kernels, detectable only at 20 and 24 DAP. The expression patterns of *Su1, ZmIso2*, and *Zpu1* transcripts closely follow the overall timing of starch accumulation in the maize endosperm, providing additional support for the role for these enzymes in starch anabolism. If DBEs were simply expressed during kernel development in anticipation for germination, it would not be predicted that expression would commence as early at 4 DAP. A hypothesis based on characterization of the barley isoamylase mutant proposed that DBEs are required for proper starch granule initiation. DBE transcripts *Su1*, *ZmIso2*, and *Zpu1* are expressed very early in kernel development (by 4 DAP), suggesting a fundamental role in the synthesis of starch that does not preclude a possible function in granule initiation.

Expression of DBEs in Germinating Seeds

During the germination of cereal grains, starch hydrolytic enzymes are secreted by the embryo and aleurone layer of the endosperm. Starch is hydrolyzed completely to glucose, which is absorbed by the scutellum, converted to sucrose, and then used by the emerging plant before it attains photoautotrophy. DBEs are the principal enzymes capable of hydrolyzing $\alpha(1\rightarrow6)$ linkages in glucose polymers. Thus, their activity during starch

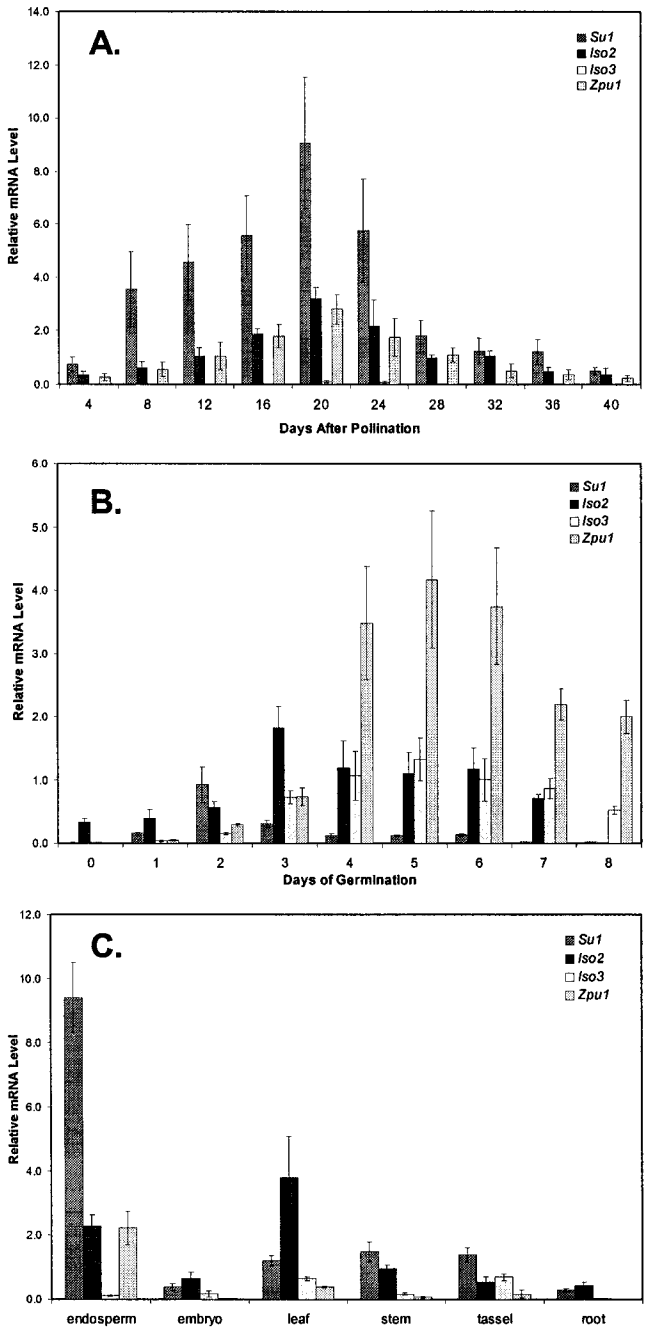

Figure 5.3. Relative quantitation of DBE gene expression. For each tissue, RNA was extracted, random hexamer-primed cDNA was synthesized, and the threshold cycle (Ct) of the 18s rRNA, *Su1*, *ZmIso2*, *ZmIso3*, and *Zpu1* sequences was determined using real-time PCR. Relative mRNA level in the sample is determined by the equation $2^{(-\Delta Ct)}$ x 1000, where $\Delta Ct = (Ct_{target} - Ct_{18s})$. Results shown are the mean ± SE of three independent samples. **A.** DBE gene expression was quantified during kernel development. **B.** DBE gene expression was measured during germination. **C.** DBE gene expression in various maize tissues was examined.

degradation would be required to make this process as efficient as possible. In addition to *de novo* synthesis of starch degradative enzymes during germination, aleurone cells secrete proteases that serve to activate some hydrolytic enzymes that have been stored from the time of kernel development.

To examine DBE transcript expression in germinating seeds, dry kernels were placed in a Petri dish on moist filter paper and incubated at 30°C. Samples were removed on successive days during the germination process, RNA was extracted, and real-time quantitative PCR analysis of cDNA was conducted for each DBE gene and the 18s rRNA. To provide a baseline for RNA molecules potentially stored from the time of kernel development, RNA was extracted from dry kernels (0 days of germination). Except for *ZmIso2*, no DBE transcripts are detectable at this time point (Figure 5.3B). The most abundant transcript throughout the process of germination is *Zpu1*, which is two-fold higher than any other DBE at days 4-6 of germination. Of the isoamylase-type DBEs, *ZmIso2* and *ZmIso3* exhibit a similar pattern of expression. *Su1* transcript is expressed at low to undetectable levels during germination (Figure 5.3B).

These data support the hypothesis that *Zpu1* is required for the efficient degradation of storage starch, consistent with evidence from mutant studies, which showed lack of *Zpu1* caused a delay in germination as well as an overall decrease in the rate of starch degradation (Dinges et al., 2003). In spite of the germination delay, pullulanase mutants are nonetheless able to emerge from the soil and grow normally. Some of the hydrolytic activity provided by *ZmIso2* and/or *ZmIso3* must be able to compensate for the loss of pullulanase-type DBE by providing $\alpha(1\rightarrow6)$ glucan hydrolase activity.

Expression of DBEs in Other Tissues

To determine the general location of DBE transcripts within the developing kernel, endosperm and embryo tissue were separated from kernels at 20 DAP. *Su1* and *ZmIso2* transcripts were located primarily in the endosperm, but were also present to some extent in the embryo tissue. Despite its low transcript abundance, *ZmIso3* appeared to be embryo-specific and not found in the endosperm. In contrast, *Zpu1* transcripts were found entirely in the endosperm portion of the developing kernel, since no *Zpu1* transcript was detected in the embryo.

In addition to the developing kernel, nearly all tissues of the plant accumulate starch at some point in their development. To see if DBEs are involved in the starch metabolism of non-storage tissues, the expression of DBE genes was examined in leaf, root, stem, and tassel. Plant leaves synthesize starch during the light portion of the diurnal cycle and subsequently degrade the starch during the dark. Leaf samples were collected at the mid-point of a 16 h day. At this time-point, *ZmIso2* is the most abundant DBE transcript, followed by reduced levels of *Su1, ZmIso3,* and *Zpu1*. A similar pattern of expression is also observed in the RNA transcripts extracted from stem tissue, except that *Zpu1* is expressed at a very low level, approaching the limits of detection.

RNA was extracted from tassel tissue at the onset of anthesis. Starch within the pollen grain provides energy for the fertilization process by supporting pollen germination and tube elongation (Datta et al., 2002). *Su1* is the most abundant DBE transcript in the tassel, with *ZmIso2* and *ZmIso3* expressed at approximately one-half the level of *Su1*. However, this analysis does not distinguish between those transcripts specifically expressed in the pollen versus those expressed in other parts of the male flower.

Since root tissues are not photosynthetic, starch is synthesized from carbon imported from the leaf tissue. The precise function of starch in root tissue is not clear. It may supply root tissues with energy when flux from the leaf is slowed or otherwise interrupted. It may also serve an important function in the gravitropic response of roots (Chen et al., 1999). In this case, it is believed that sedimentation of amyloplasts serves as the orienting signal to which the plant responds. Since starch is metabolized in these tissues, there is clearly a role for the debranching enzymes in its synthesis and degradation. *Su1* and *ZmIso2* transcripts are detectable in these tissues, but *ZmIso3* and *Zpu1* transcripts are undetectable by real time quantitative PCR.

Conclusion

To further describe the function(s) of debranching enzymes in the metabolism of starch, this study identified all of the genes that are potentially contributing to the $\alpha(1\rightarrow6)$ glucan hydrolase activity of the maize plant. The complete genome sequences of rice and Arabidopsis, and cloned cDNAs from potato and maize have shown that plants possess three isoamylase-type DBE genes and one pullulanase-type DBE gene. Phylogenetic analysis has shown that each sequence represents a distinct and evolutionarily conserved form. DBEs are expressed during periods of both net starch synthesis and degradation. Hence, further evidence is provided that these enzymes perform a dual function, since their mRNA transcript expression patterns matches those periods where there is both synthesis and degradation of starch.

Previous biochemical studies of the isoamylase from developing potato tubers have shown that the *StIsa1* and *StIsa2* forms may interact in a multimeric enzyme complex.

Consistent with this hypothesis, the overlapping expression patterns of *Su1* and *ZmIso2* during kernel development suggests that an analogous situation may occur in maize endosperm. These two forms may function together to support the biosynthetic function of isoamylase-type DBE. In contrast to this "biosynthetic complex", *ZmIso3* and *ZmIso2* exhibit a similar pattern of expression during germination. These two forms may interact to form a "degradative complex" that performs the catabolic function of isoamylase-type DBE. Thus, we propose that isoamylase isoforms may assume altered assembly states, with different isoform components that depend on the particular metabolic function, i.e. starch synthesis or degradation.

In the maize kernel the synthesis and degradation of starch are spaced temporally between the times of kernel development and germination, which are naturally separated by many months. The expression of the components of both biosynthetic and degradative machineries would be expected to be confined to those periods when they are required. However, in other tissues, such as the leaf or the tassel, the temporal separation of synthesis and degradation is greatly reduced, on the order of a few hours. The DBEs functioning in both synthesis and degradation might need to be expressed at the mRNA level throughout this process, such that they are available for subtle and responsive metabolic flux changes, requiring their activity. In such cases, these responses would likely be controlled at the post-translational level, perhaps involving the reorganization of protein complexes from the assembly to disassembly states.

Materials and Methods

Plant Materials

All tissues used in this study were isolated from plants of maize (*Zea mays* L.) inbred W64A. For tissues harvested at various days after pollination, plants were grown under field conditions in Ames, IA during the summer of 2002. For tissues harvested at various days after germination, seeds were placed in a Petri plate on moist filter paper at 30°C. The cotyledon and roots were removed at the time of collection, but the scutellum and aleurone layers were left intact. Leaf, stem, root, and tassel tissues were isolated from greenhouse-grown plants. Tissues were quick-frozen in liquid nitrogen and placed at -80°C until used.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using the Concert Plant RNA Extraction Reagent (Invitrogen, Catalog No. 12322-012), according to the manufacturer's instructions. Approximately 1 μg RNA was treated with DNAse (Promega, Madison, WI) in a total volume of 10 μL, for 30 min at 37°C, followed by heat inactivation at 65°C for 10 min. Random hexamer-primed cDNA synthesis of the DNAse-treated RNA was performed using the Taqman Reverse Transcription Reagents (Applied Biosystems, Catalog No. N808-0234) in a total volume of 100 μL. cDNA was aliquoted and stored at -20°C until use.

Isolation of *ZmIso2* and *ZmIso3* Sequences

Total RNA was isolated from W64A kernels at 20 DAP. RT-PCR was performed using the Superscript One-Step RT-PCR system (Invitrogen, Catalog No. 10928-034). Primers JD37 (5'-AAA GGA CCT TAT TTY CCN TAY CA-3') and JD42 (5'-CCA CAT TCA TCT CCC ATR TTN AG-3') amplified the central portion of the *ZmIso2* cDNA.

Primers JD21 (5'-CAT CAC GAC CAC CTT CTC CAT TTG-3') and JD24 (5'-GAA CTT GGC GTT AAY GCN GTN GAR C-3') amplified the central portion of the *ZmIso3* cDNA.

5' and 3' rapid amplification of cDNA ends (RACE) was performed using the GeneRacer RACE Kit (Invitrogen, Catalog No. L1502-01). 5' RACE of *ZmIso2* was performed with the oligonucleotide JD62 (5'-CAT TCT TCA AGG ACG CGA GAC ACC TTA-3'), and 3' RACE was performed using JD48 (5'-TCA AAC CAG GCT AAG ACA GAT AC-3'). Similarly, the 5' end of *ZmIso3* was obtained using RACE primer JD28 (5'-AGC CTC ATT TGT ATG GTT GTA AA-3'). The 3' sequence information for *ZmIso3* was contained in an EST clone provided by Pioneer Hi-Bred Intl.

Phylogenetic Analysis

Putative plant and bacterial DBE proteins were identified by BLAST searches in Genbank. Protein sequences were aligned using Pileup (Genetics Computer Group, Madison, WI). Phylogenetic analyses were performed using maximum parsimony implemented with the PAUP (Phylogenetic Analysis Using Parsimony) program. Bootstrap resampling analyses were performed to assess the degree of support for each branch on the phylogenetic tree.

Real-time Quantitative PCR

Real-time PCR was performed with specific oligonucleotide primers for each maize DBE gene and the 18s rRNA (Table 5.1) using the Applied Biosystems 5700 sequence detection system. Reactions were performed in a 50 μL volume including the cDNA sample, 100 nM oligonucleotide primers, and 1X of SYBR Green PCR Master Mix (Applied Biosystems, Catalog No. 4309155), which includes Amplitaq Gold DNA Polymerase,

Table 5.1. Primer sequences for Real-Time PCR

Gene Name	Accession No.	Primer Name	Oligonucleotide Sequence	cDNA Position
18s rRNA	AF168884	Zm18sF	5′-GCCTTCGGGATCGGAGTAAT-3′	848 - 867
		Zm18sR	5′-CCCCCAACTTTCGTTCTTGA-3′	998 - 978
ZmIso1/Su1	U18908	Su1RTF	5′-TTCGTTGCCTTCACCATGAA-3′	2187 - 2206
		Su1RTR	5′-CCGGAAGGTGACTGGTGTTG-3′	2259 - 2240
ZmIso2	AY172633	Iso2RTF	5′-GCAACCATCCAGTGACTCAGAA-3′	1428 - 1449
		Iso2RTR	5′-CATGGAAGTCGAGCACCCA-3′	1493 - 1475
ZmIso3	AY172634	Iso3RTF	5′-CGCCCATGAGTCGTTATGCTA-3′	1118 - 1138
		Iso3RTR	5′-CAGAATTATGAAATGCCTTGACCA-3′	1212 - 1189
Zpu1	AF080567	Zpu1RTF	5′-CGTACGACACCGCCACAG-3′	2861 - 2878
		Zpu1RTR	5′-GATGCCGAATGCACTTGCT-3′	2972 - 2954

buffer, dNTPs, and 2 mM $MgCl_2$. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, 60°C for 1 min. Fluorescence data were collected using the Sequence Detector Software (version 1.3), and the threshold cycle (Ct) for each sample was calculated by determining the point at which fluorescence exceeded the threshold limit, corresponding to the exponential phase of the PCR reaction ($\Delta R_n = 0.1$). Amplification plots and predicted Ct values from the exponential phase of the PCR were exported directly into Microsoft Excel for analysis.

Primers, Control, and Relative Quantitation

Gene-specific oligonucleotides (Table 5.1) were designed using the Primer Express software (Applied Biosystems). Care was taken to select primers in regions of the sequences that were specific to each isoform. Before the analysis of the cDNA samples, the efficiency

of each set of primers was determined. A standard curve for each primer pair was generated using a representative cDNA sample (W64A Kernel at 20 DAP) diluted at two-fold intervals. Plotting of Ct versus log cDNA showed that the amplification with a given primer pair was linear with respect to the log concentration of cDNA. Standard curves generated for the gene-specific primer pairs with slopes that were approximately equal to the standard curve for the 18s rRNA were chosen for use in the relative quantitation experiments, since direct comparisons could then be made between Ct values. All standard curve and sample assays were performed in triplicate, so that the accuracy of the quantitation could be verified between wells of the reaction plate. The specificity of the primers was verified using melting curve analysis (dissociation kinetics), agarose gel electrophoresis, and DNA sequencing. No template control (NTC) assays were performed for each primer set used, which produced negligible signal detection, typically 38-40 Cts in value. All DBE gene-specific primer sets used for analyzing the cDNA samples typically produced 20-35 Cts in value. cDNA samples where diluted 100-fold prior to detection of 18s rRNA sequences, which then typically produced 14-20 Cts in value.

In one experiment, at least two values, corresponding to the relative transcript level, were produced for each sample. The experiments were repeated at least three times from independently extracted tissue samples, and the data averaged. All results shown indicate the relative mRNA level ± standard error (SE). The relative mRNA level is expressed in arbitrary units according to, mRNA level = $(2^{-\Delta Ct})$ x 1000, where $\Delta Ct = (Ct_{target} - Ct_{18s\ rRNA})$.

Acknowledgements

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CHAPTER 6. SUMMARY AND GENERAL CONCLUSIONS

General Discussion

The overall goal of this project was to identify and characterize the function(s) in carbohydrate metabolism of all maize starch debranching enzyme genes. Prior to this study, two starch debranching enzyme genes were identified, *Su1* and *Zpu1*. In view of that, the first research objective was to identify all other maize genes coding for α(1→6) glucan hydrolase proteins. Using a combination of gene discovery and degenerate PCR procedures, two additional isoamylase cDNAs were cloned from maize, *ZmIso2* and *ZmIso3* (Chapter 5). The maize starch DBE gene family was therefore found to contain four genes coding for three DBEs of the isoamylase-type (*Su1, ZmIso2,* and *ZmIso3*), and one DBE of the pullulanase-type (*Zpu1*). Consistent with information from the complete genome sequences available for Arabidopsis and rice, these four genes likely represent all of the α(1→6) glucan hydrolase coding sequences in the maize genome.

The second major objective of this research was to determine the specific role played in starch anabolism and catabolism by the proteins coded for by each DBE gene. An approach involving the characterization of genetic mutants was used to meet this objective. To describe the function of the isoamylase-type DBE isoform *Su1*, the allelic diversity of this locus was used as a tool to link molecular genetic defects to changes in endosperm carbohydrate composition and pleiotropic effects on other starch metabolizing enzymes (Chapter 2). The readily apparent kernel phenotype of many *su1* mutants facilitated the identification of alleles with a range of phenotypes. The data showed that the predicted consequences of the molecular defects were related to the phenotypic severity of the allele.

Nevertheless, the residual isoamylase activity (or the lack thereof) in the mutants was not the sole factor in determining the severity of the phenotype. It was observed that the presence or absence of a polypeptide, albeit non-functional, played an important part in determining the different pleiotropic effects on other starch metabolizing enzymes. Interactions between starch metabolizing enzymes are likely to be an important functional requirement for synthesis of starch with the necessary molecular structure (described further below).

The severity of the *su1*- defect also had significant effects on the structure of residual amylopectin (Chapter 2). A marked increase in short linear glucan chains relative to the wild-type was observed in the "sugary" amylopectin. Consistent with the results of studies in rice (Nakamura et al., 1996; Nakamura et al., 1997), these data suggest that the SU1 isoamylase is functioning during the time of starch biosynthesis and acting on an amylopectin precursor prior to crystallization. Consequently, the data support the "glucan trimming model," which proposes that DBEs are involved in removing extraneous branches from amylopectin during starch synthesis (Ball et al., 1996). This is in contrast to the WSP clearing model, which does not predict that DBEs would act on an amylopectin precursor. According to this hypothesis, no change in amylopectin structure would be expected to result from DBE deficiency, since the model implies that DBEs act only on soluble glucan in the stroma (Zeeman et al., 1998). There is a possibility that pleiotropic effects on SS and/or SBE in *su1* mutants was the cause of the change in amylopectin structure and that isoamylase was not directly involved. However, the pleiotropic effects on detectable starch metabolizing enzymes did not correlate with the observed structural changes, whereas the direct loss of isoamylase-type DBE correlated well with these observations.

Because of the dramatic effects that isoamylase mutations have on starch biosynthesis, it is difficult to ascertain the exact role of the isoamylase-type DBE isoform in starch degradation. Since *Su1* transcripts are not expressed during kernel germination (Chapter 5), this isoform probably does not contribute significantly to glucan hydrolysis during the degradation of storage starch. Rather, *ZmIso2* and *ZmIso3* appear to be the principal isoamylase-type DBE isoforms expressed during germination. Characterization of mutants of these genes will more fully clarify the role of these isoforms in starch degradation.

To describe the role of the pullulanase-type DBE in maize starch metabolism, reverse genetics identified a null mutation of the maize pullulanase gene *Zpu1* (Chapter 3). Detailed characterization of this mutation indicated that the pullulanase-type DBE is important for degradation of storage starch during germination. Starch catabolism was clearly impaired, but not irreparably so by the *zpu1-204* mutation since kernels were able to germinate following a delay. The degradation of transient leaf starch was also affected by the *Zpu1* knockout mutation. This interpretation of these data was complicated by the observation that starch synthesis in the leaf is affected by the *zpu1-204* mutation. The defect in starch synthesis was likely the result of reduced SBEIIa activity in the pullulanase mutant. The pleiotropic loss SBEIIa activity cannot entirely explain the alteration in starch synthesis however, since the effects are different from those observed in mutants specifically affected in gene coding for SBEIIa. In spite of this, the starch that did accumulate in *zpu1-204* leaves was degraded more slowly than wild-type.

Singly mutant *zpu1-204* kernels did not exhibit major changes in carbohydrate composition, but do contain WSP in the form of small molecular weight branched MOS,

suggesting a function for ZPU1 in starch synthesis. Since the MOS did not accumulate to a significant extent, at least one of the isoamylase-type DBE isoforms must be able to degrade this material before it attains the size and/or quantity normally found in phytoglycogen. Moreover, an overlapping role for ZPU1 with SU1 during starch synthesis in the kernel was indicated by analysis of double mutants (Chapter 3, 4). When *zpu1-204* was put in combination with several different *su1-* mutant alleles, there was an increase in the amount of phytoglycogen produced in these kernels, such that phenotypic differences were readily apparent. However, when *zpu1-204* was put in combination with the null *su1-4582* allele, there was no additional phytoglycogen that accumulated and some granular starch remained. These results suggest that the accumulation and MOS and phytoglycogen might be the consequences of loss of degradative activity in the stroma of the plastid, consistent with a role for DBEs described by the WSP clearing model (Zeeman et al., 1998).

These data also show that the defect in SU1 is the primary cause of the phytoglycogen-accumulating phenotype, but ZPU1 can compensate partially to prevent some phytoglycogen accumulation. Some SU1 activity and/or protein are required for the biosynthetic function of ZPU1 since when the SU1 isoamylase was completely missing, the pullulanase was unable to compensate to any extent. These data are consistent with the results observed in rice, which showed that pullulanase can be important for starch accumulation in some sugary backgrounds (Kubo et al., 1999). The data also indicate that some starch can be synthesized, even in the absence of detectable DBE activity.

The results from this and other studies of DBEs, provides some evidence that there are two separate and perhaps mutually exclusive functions of DBEs during the synthesis of starch. *Su1* mutants of maize and rice accumulate phytoglycogen with concomitant changes

in amylopectin structure. However, differences in amylopectin structure are not seen in isoamylase mutations of barley and Arabidopsis. One explanation for this observation is that the trimming function is compensated for by another DBE isoform in these species. Nevertheless, these findings demonstrate that it is possible to accumulate phytoglycogen without changing amylopectin structure. Conversely, altered DBE expression may result in changes in amylopectin structure without the accumulation of phytoglycogen. Transgenic maize plants overexpressing *Su1* or expressing an antisense form frequently exhibit an altered chain length distribution without the accumulation of phytoglycogen (T.A. Bierwagen, A.M. Myers, and M.G. James, unpublished). Taken together, this information suggests that DBEs may participate in the direct and indirect functions described by both the glucan trimming and WSP-clearing models.

Genetic and biochemical studies have led to the hypothesis that SU1 is involved in a multimeric protein complex, likely to involve other proteins that similarly recognize glucose polymers as substrates. The data presented here have also demonstrated the significance of enzyme interactions to the process of starch biosynthesis. Mutants in both DBE isoform genes *Su1* and *Zpu1* pleiotropically affected several starch metabolizing enzymes, including SBE, α-amylase, β-amylase, and other DBEs. The effects of *su1*- mutations on other starch metabolizing enzymes reveal that all of these proteins are likely acting in a concerted manner during the biosynthesis and degradation of starch. Studies to demonstrate direct physical interaction between several of these proteins are currently underway.

The SU1 protein is postulated to assemble in a complex, consisting of multiple subunits of the SU1 protein. This was indicated from reports that the rice SU1 assembles as a homomultimer of 4-6 subunits (Fujita et al., 1999). Characterization of the potato

isoamylase, however, has indicated that the isoamylase complex exists as a heteromultimer. The StISA1 (SU1) protein was physically associated with the StISA2 form and the combination of these two proteins showed altered activities when tested on different substrates (Hussain et al. 2003). This observation is supported by study of the *Chlamydomonas* isoamylase, in which two genetic loci were found to contribute components to the isoamylase complex (Dauville et al. 2001). The data presented in Chapter 2 and Chapter 5 support the hypothesis that the maize isoamylase also forms a multimeric complex. The dominance/recessiveness of several *su1-* alleles was investigated in heteroallelic combinations. Alleles that produce an inactive protein are dominant to alleles that produce reduced amounts of a functional protein, suggesting that the inactive protein interfered with the organization of the multimeric complex in some way. The real-time quantitative PCR expression analysis showed that *Su1* and *ZmIso2* have similar expression patterns during kernel development, consistent with the idea that these two forms may join in a heteromultimer. Moreover, during the time of starch degradation during germination, the pattern of *ZmIso2* and *ZmIso3* expression was very similar, suggesting that these two forms might interact to form an isoamylase complex with different substrate specificity or activity designed for degradation of starch rather than its synthesis. Thus, the subunit composition of the isoamylase may be different depending on the particular metabolic state of the cell.

Recommendations for Future Research

This study has found that starch DBEs are involved in dual aspects of starch metabolism, both in the synthesis and degradation of amylopectin. A function for the pullulanase-type DBE isoform *Zpu1* in starch degradation in the leaf and germinating seed

was indicated, as well as a partial overlapping function with the isoamylase-type DBE *Su1* in the clearing of soluble glucan and the prevention of phytoglycogen accumulation. The data presented here have led to the conclusion that DBEs are involved in starch biosynthesis performing functions described by both the glucan trimming and WSP clearing models.

The precise function of *ZmIso2* and *ZmIso3* isoforms remains to be determined. This will most reasonably be accomplished using a genetic approach similar to that described for *Zpu1* in Chapter 3 of this dissertation. Indeed, reverse genetics has already been used to identify transposable element insertions in *ZmIso2* and *ZmIso3* (see Appendix), but a detailed characterization of the effects of these mutations remains to be conducted. Generation of double and triple isoamylase mutants will further demonstrate the importance of this family of enzymes to starch metabolism in the maize plant.

Biochemical studies should be conducted to investigate the hypothesis that a threshold of isoamylase activity and/or protein is required to prevent phytoglycogen accumulation. Expression of recombinant *su1-* mutant proteins will determine whether or not these modified isoamylases possess residual catalytic activity. This will further describe the complex allele-specific effects that are observed in the *su1-* alleles with the range of phenotypes described in Chapter 2.

To further describe the nature of the isoamylase in maize tissues, biochemical purification of the native complex will help to identify the subunit composition during both kernel development and germination. Specific antibodies to the three isoamylase-type DBE isoforms will test whether this complex exists as a homomultimer or heteromultimer. Additional experiments to test protein interactions, such as yeast two-hybrid and affinity chromatography will also help determine whether these proteins can physically interact.

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APPENDIX.

REVERSE GENETICS OF *ZmIso2* and *ZmIso3*

To characterize the metabolic function(s) of the isoamylases-type DBEs coded for by *ZmIso2* and *ZmIso3*, reverse genetics was used to identify knockout mutations in these genes, with the idea that loss of function might reveal the role of these isoforms in starch metabolism. The Pioneer TUSC system (Trait Utility System for Corn) was used to identify *Mutator* transposable element insertions in the *ZmIso2* and *ZmIso3* genes. TUSC F_1 maize (*Zea mays* L.) seeds were generated by Pioneer Hi-Bred International, Inc. (Johnston, IA) by crossing a *Mu*-active line (i.e. possessing a high *Mutator* copy number and the autonomous element *MuDR*) to a non-*Mutator* line. PCR screening of DNA pools was performed on DNA isolated from the leaves of the F_1 plants and they were self-pollinated to give F_2 seed. To confirm the heritability of positive insertion signals identified from amplified F_1 pools, F_2 genomic DNA was isolated from dry kernels and the PCR screening repeated. Consequently, F_2 seed from plants possessing germinal insertions in *ZmIso2* and *ZmIso3* were provided by Pioneer.

Oligonucleotide primers 63550 (5′-ATG GGC TAG CTG TGC CAT CCA TAA GG-3′) and 63553 (5′-ATA TGA GGA AAA TCC TAG CAC CTG GGG TG-3′) amplified a 1592 bp product in *ZmIso2* cDNA, and correspondingly, detects the wild-type allele. *Mu* insertions in *ZmIso2* were detected with primers 63553 and the *Mu*-terminal inverted repeat (TIR) primer 9242 (5′-AGA GAA GCC AAC GCC AWC GCC TCY ATT TCG TC-3′) Forward primers 63558 (5′-ATT TGA TGC CTT GCG GAG TAC CAC GAC-3′) or JD64 (5′- AAG GTG AGA TCA GCA TCT CCG TCT CTT-3′), together with the reverse primer

63557 (5′-AAT AGC TGG AGC AAG CCT GCT TGA CTC-3′) amplified a ~2000 bp product from the *ZmIso3* gene. Primers 63557 and 9242 were used to detect *Mu* insertions in the corresponding region of the gene.

Since *ZmIso2* contains no introns, primers for TUSC pool screening that were designed for the coding region had a high probability of identifying true knockout insertions. In contrast, the fragmented gene structure of *ZmIso3* (20 predicted exons) meant that there was a high likelihood of *Mu* insertion in an intron. Two independent germinal insertion alleles were identified by Pioneer in the *ZmIso2* gene. These insertions were subsequently mapped to nt position 882 and 950 of the *ZmIso2* gene (Genbank Accession No. AY17633), and the alleles have been named *zmiso2-339* and *zmiso2-347*, respectively, with the allele number corresponding to the TUSC pool from which these plants were identified (Figure A.1A). Both of these insertions are predicted to be null alleles since they disrupt the protein coding region of the *ZmIso2* gene.

Two heritable *Mu* insertions in *ZmIso3* were identified from the TUSC pool screening (Figure A.1B). However, the prediction that these insertions would alter gene expression is not as clear as the insertions identified for *ZmIso2*. The allele *zmiso3-233* contains a *Mu* element at nt position 67 of the *ZmIso3* cDNA (Genbank Accession No. AY17634), predicted to be in the 5′-untranslated region (UTR). It is likely that insertion at this position would result in a *Mu*-suppressible phenotype, in which transcription proceeds from the *Mu* element itself. The *zmiso3-688* allele possesses an insertion of the transposon in the promoter region 85 bp upstream of the transcription initiation site. The *zmiso3-688* allele may exhibit *Mu*-suppression or not result in any detectable changes in gene expression. Thus, the probability of generating a true knockout mutation for *ZmIso3* may be improved

by screening for small sequence deletions adjacent to the *Mu* element or germinal excisions in these plants.

A detailed characterization of mRNA and/or protein accumulation remains to be conducted for the mutant alleles of *ZmIso2* and *ZmIso3* that have been identified. Following several generations of this backcrossing, thorough biochemical studies regarding starch and/or phytoglycogen accumulation in the leaf and endosperm will be performed.

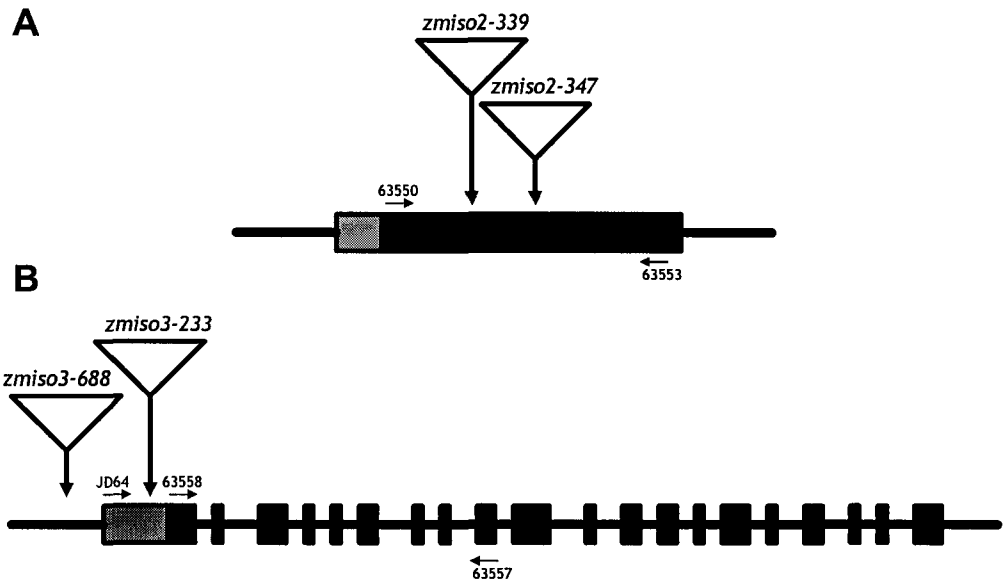

Figure A.1. Molecular structure of *ZmIso2* and *ZmIso3* mutant alleles. **A.** Relative position of *Mu* insertions in the *ZmIso2* gene are indicated by the open triangles. **B.** Relative position of *Mu* insertions in the *ZmIso3* gene are indicated by the open triangles. Transcribed regions are indicated by the black boxes. The 5′ UTR is indicated by the gray box.

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